

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

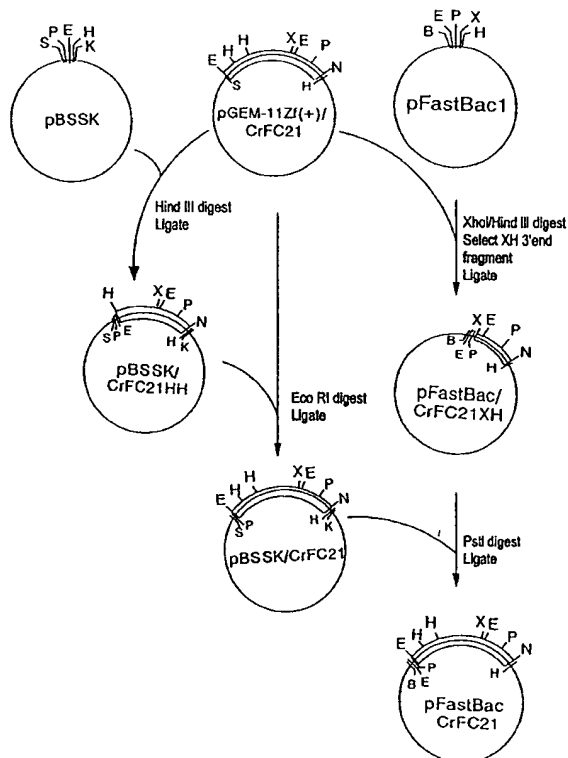
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/63, 15/55, C12Q 1/37	A1	(11) International Publication Number: WO 99/15676 (43) International Publication Date: 1 April 1999 (01.04.99)
(21) International Application Number: PCT/SG98/00073 (22) International Filing Date: 18 September 1998 (18.09.98) (30) Priority Data: 60/058,816 19 September 1997 (19.09.97) US 09/081,767 21 May 1998 (21.05.98) US (71) Applicant: NATIONAL UNIVERSITY OF SINGAPORE [SG/SG]; 10 Kent Ridge Crescent, Singapore 119260 (SG). (72) Inventors: DING, Jeak, Ling; Industry & Technology Relations Office (INTRO), National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260 (SG). HO, Bow; Industry & Technology Relations Office (INTRO), National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260 (SG). (74) Agent: APPLIED RESEARCH CORPORATION; Kent Ridge, P.O. Box 1016, Singapore 911101 (SG).		(81) Designated States: SG, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: A NOVEL GENERATION OF CLONED HORSESHOE CRAB RECOMBINANT FACTOR C FOR DETECTION AND REMOVAL OF ENDOTOXIN

(57) Abstract

The horseshoe crab, *Carcinoscropsius rotundicauda* Factor C cDNA (CrFC21) has been cloned into a shuttle baculoviral vector. The recombinant baculoviral DNA was then transfected into the insect cells for expression of recombinant Factor C. Recombinant Factor C was found to be immunoreactive and is capable of binding both free and bound/immobilized lipid A. It is enzymatically active when triggered by LPS. The rFC is probably of the two-chain form, being cleaved into the heavy and light chains after activation by Gram negative bacterial endotoxin. As low as 0.01 pg (0.001 ng/ml) of LPS was detectable by the rFC, thus, indicating its potentials as a novel generation of "limulus amoebocyte lysate".



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

A Novel Generation of Cloned Horseshoe Crab Recombinant Factor C for Detection and Removal of Endotoxin

FIELD OF THE INVENTION

5 The present invention relates to recombinant Factor C (rFC) of a horseshoe crab, produced in an insect cell system. The invention also relates to vectors for producing the protein by recombinant DNA methods and to methods for using the recombinant Factor C to detect endotoxins in a sample or for removal of endotoxins from a sample by affinity methods.

THE RELATED ART

10 The amoebocytes of horseshoe crabs contain an efficient coagulation cascade system which is activated by endotoxin, also known as lipopolysaccharide (LPS) from Gram negative bacteria. The enzymatic components of the coagulation cascade and the molecular events responsible for the subsequent gelation of the amoebocyte lysate have been characterized in *Tachypleus tridentatus*¹ and *Carcinoscorpius rotundicauda*^{2,3,4}.
15 Factor C has been shown to be the intracellular endotoxin-sensitive serine protease that initiates the coagulation cascade system⁵.

By spiking, the LAL test detects femtogram levels of LPS⁶. Owing to its extreme sensitivity, the amoebocyte lysate, in particular, the *Limulus* amoebocyte lysate (LAL) has been developed into a commercial assay for widespread use in the detection of
20 pyrogenic LPS in drugs and other pharmaceutical products^{7,8}. This assay is based on the LPS-induced coagulation reaction of the lysate, culminating in formation of a gel clot. However, (a) the possible lack of specificity due to 1-3 β -D glucan and (b) the batch-to-batch variation in the sensitivity of commercial lysate to LPS, due to seasonal and

geographical differences in the starting material⁹ has prompted our laboratory to employ recombinant DNA technology to genetically-engineer Factor C as an alternative source of novel "limulus lysate" for endotoxin detection.

cDNAs encoding Factor C have been cloned^{1,10,15}. There are six potential glycosylation sites in the amino acid sequence of the Factor C from *Carcinoscorpius rotundicauda* (CrFC)^{10,15}. Cloned cDNA encoding CrFC has been expressed in *E. coli*¹¹ and also in yeast expression systems^{12, 24}. The rFC obtained from yeast was found to be immunoreactive and capable of binding LPS, although only limited amounts of rFC produced in yeast were soluble^{13,14}. Also, it was found that LPS could not activate the enzymatic activity of yeast rFC, thus, a direct enzyme-based LPS detection is not possible using rFC produced in yeast¹⁴.

SUMMARY OF THE INVENTION

The present inventors believed that expression in insect cells rather than in a prokaryotic or simple eukaryotic expression system is suitable for producing rFC with full biological activity. Furthermore, horseshoe crabs and insects belong to the same phylum, Arthropoda, and so insect cells might more closely resemble the cells of the horseshoe crab than yeast cells in their physiology and biochemistry. Thus, rFC produced in insect cells might more closely resemble the protein as purified from the horseshoe crab and retain the bioactivity of having a serine protease activity activated by LPS.

The present invention relates to genetic engineering of a bioactive rFC, which unequivocally exhibits full biological functionality. It is capable of specifically recognizing and binding LPS and lipid A in both free and immobilized forms. Interference from 1-3 β -D-glucan, which switches on the alternate pathway in the

coagulation cascade in conventional LAL, is not anticipated in assays of the present invention that use only Factor C as the LPS-binding, serine protease enzyme. Both the LPS-activated enzymatic assays of rFC and the ELISA lipid A binding assay could be formulated into a rapid high throughput mass screening test for LPS. Thus, a novel generation of "limulus amoebocyte lysate" has been invented, being capable of rapid and sensitive diagnosis and removal of subpicogram levels of endotoxin. The invention provides a standardized and convenient source of enzyme-based diagnostic reagent for detection of the ubiquitously contaminating endotoxin in pharmaceutical products. This inexhaustible supply of genetically-engineered Factor C can be easily standardized to circumvent batch-to-batch variations in sensitivity to LPS, a problem faced by the conventional LAL industry. Furthermore, the ability of the rFC of the invention to protect mice from endotoxemia, as well as its bacteriostatic activity, adds to its value in *in vivo* applications. Furthermore, the availability of rFC obviates the need for routine harvesting of the horseshoe crab for procurement of their amoebocyte lysate, and therefore, conserves this endangered "living fossil".

The present inventors have succeeded in expressing biologically active rFC using recombinant baculoviruses and insect host cells. The rFC obtained is enzymatically active. Thus, expression of rFC in insect cells is a convenient and economical source of rFC protein for use in rapid, sensitive, specific and quantitative determination of LPS in pharmaceutical products and other biological fluids.

Thus, the present invention comprises purified rFC that is enzymatically active. The phrase "enzymatically active" means that the Factor C protein has the biological activity of binding LPS or lipid A, being activated as to its serine protease activity upon LPS or lipid A binding. Enzymatically active rFC will induce coagulation of an

amoebocyte lysate and will also cleave synthetic substrates such as, but not limited to, Boc-Val-Pro-Arg-MCA, Mu-Val-Pro-Arg-AFC and Boc-Val-Pro-Arg-pNA.

The present invention is also embodied in a method for producing substantially purified, enzymatically active rFC. The method comprises expressing DNA encoding a Factor C protein having the enzymatic activity described above in a culture of insect cells, then isolating the enzymatically active Factor C protein. The isolation preferably includes an ultrafiltration step. The purification preferably also includes a step of gel-filtration chromatography on a matrix having an exclusion limit of 100 kilodaltons. The gel filtration is preferably applied after the ultrafiltration. The exclusion limit of the gel filtration matrix can vary substantially; an effective matrix will provide at least about a 4-fold increase in the serine protease activity of an ultrafiltered crude preparation as measured by the fluorometric assay described herein.

The present invention also encompasses host-vector systems for expressing enzymatically active rFC. The host cells in these embodiments of the invention are insect cells, preferably lepidopteran cells. The vectors in these embodiments support replication of inserted DNA in insect cells and expression of heterologous DNA in insect cells. The vectors are preferably baculovirus or plasmid vectors. The heterologous DNA is sufficient to encode a Factor C enzyme of a horseshoe crab, preferably of the genus *Carcinoscorpius*, *Tachypleus* or *Limulus*. Preferred heterologous DNA is a polynucleotide having the sequence shown in SEQ ID NO:1 or SEQ ID NO:3.

The present invention is also embodied in assays for endotoxin comprising contacting a sample to be assayed for the presence of endotoxin or LPS or Lipid A with enzymatically active rFC according to the invention and measuring the serine protease activity of the rFC. The amount of serine protease activity of the rFC will reflect its activation due to binding of LPS or Lipid A or of another endotoxin known in the art to

bind to Factor C of a horseshoe crab. The serine protease activity is conveniently measured by any method known in the art but is preferably measured by a chromogenic or fluorogenic method. In such a method formation of a product from a substrate by cleavage of the substrate by the serine protease activity of the rFC, resulting in a change
5 in color or in fluorescence emission, is measured. Preferred substrates for such a chromogenic or fluorogenic assay are N-t-BOC-Val-Pro-Arg-MCA, Mu-Val-Pro-Arg-AFC and Boc-Val-Pro-Arg-pNA.

Additional embodiments of the invention include immunologic methods for assaying the presence of Lipid A or LPS or endotoxin in a sample. These methods of the
10 invention rely upon binding of antibody that specifically binds to Factor C and subsequent detection or quantitation of the amount of the Factor C-antibody complex. In a preferred embodiment, the sample to be assayed is contacted with immobilized antibody that specifically binds to Lipid A or LPS or endotoxin as the ligand to form immobilized ligand. The immobilized ligand is then contacted with rFC according to the
15 present invention to form immobilized rFC. Then the immobilized rFC is contacted with a second antibody that specifically binds the rFC. Finally, the presence or preferably the amount of the rFC-second antibody complex is determined. This determination can be performed by any method typical in the art such as a third antibody that binds the second antibody, perhaps through its Fc portion, or the like. In an alternate embodiment of this
20 aspect of the invention, the second antibody is omitted and the enzymatic activity of the immobilized rFC is measured.

In another embodiment of the invention, the specific binding of LPS or lipid A to rFC is employed in a BIACORE™ assay (Pharmacia Biotech). By immobilizing the rFC on the substrate plate of the BIACORE™ apparatus, the presence of LPS or lipid A in a
25 sample can be detected. Optimization of the amount of the rFC to be immobilized for a

given load of LPS in a sample is considered within the skill of the ordinary practitioner. The BIACORE™ apparatus is operated in accord with the manufacturer's instructions.

Also, the present invention is embodied in methods for removal of endotoxin from a sample, wherein immobilized rFC is contacted with the sample, under conditions such that endotoxin in the sample binds the immobilized rFC, then the bound endotoxin is
5 separated from the sample.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1A-B: Cloning of CrFC21 cDNA into the baculovirus expression vector, pFastBac I™. Two constructions of the same plasmid were done. (1A) A 2.3 kb
10 Hind III fragment from pGEM11Zf(+)/CrFC21¹⁵ was cloned into HindIII linearized pBluescript™ SK(+) (pBSSK) to give pBSSK/CrFC21/HH. This was then digested with Eco RI and ligated with the 2.3 kb Eco RI fragment from pGEM11Zf(+)/CrFC21 to regenerate recombinant pBSSK/CrFC21. Separately, a 1.3 kb Xho I/Hind III fragment, derived from pGEM11Zf(+)/CrFC21, was cloned into XhoI/HindIII digested pFastBac
15 I™ to give pFastBac/CrFC21XH. The final full-length construct, pFastBac/CrFC21 was generated when the 2.9 kb Pst I CrFC fragment from pBSSK/CrFC21 was ligated to Pst I linearized pFastBac/CrFC21/XH.

(1B) The pFastBac I™ vector and an intermediate CrFC subclone of pGem11Zf(+)/CrFCEN were digested with Eco RI and Hind III. The liberated CrFCEN
20 insert was ligated directionally into the linearized pFastBac I™ vector to yield pFastBac/CrFCEN. pFastBac/CrFCEN was then digested with Eco RI, as was another vector comprising DNA encoding Factor C, pGEM11Zf(+)/CrFCEE. The insert released from pGEM11Zf(+)/CrFCEE was ligated into the linearized pFastBac/CrFCEN. The clone having the proper orientation was selected by restriction analysis and designated
25 pFastBac/CrFC21'.

Figures 2A-2D: Immunoblot analysis of Sf9 rFC. (2A) Reducing SDS PAGE of 5 µg total protein of cell lysate and culture supernatant harvested at 24 and 48 h post-induction (p.i.). (2B) Comparison of reducing and non-reducing SDS PAGE analyses of rFC from 72 h p.i. The results indicate that rFC is probably a double-chain form of Factor C as further proven by the LPS-treated (lane 2) and -untreated (lane 1) rFC under reducing (2C) and non-reducing (2D) conditions. Western Blots (Figs. 2C and 2D) were developed using a horseradish peroxidase system.

Figures 3A-3C: ELISA lipid A-binding assay of rFC. (3A) A gradation of increasing intensity of color development of the enzymatically-hydrolyzed product is seen from 0.01 to 200 ng lipid A. Rows 1 & 2 contain 10 µg total protein per well of culture supernatant from pFastBac/CrFC21 infected Sf9 cells after 72 h p.i. Rows 3 & 4 are controls containing 10 µg total protein per well of culture supernatant from wild-type ACMNPV-infected Sf9 cells after 72 h p.i. (3B) The histogram illustrates a quantification of the lipid A based on the absorbance at OD_{405nm} of 72 h culture supernatant (10 µg) and cell lysate (20 µg) after their reaction with 0.01 to 200 ng of lipid A. The results were normalized with wild type baculovirus infected samples. The culture supernatant consistently showed higher efficacy of binding lipid A even though a lower total amount of protein (10 µg) was used. (3C) Blocking of excess sites with 0.2% BSA effectively removed the non-specific background binding, and results in higher net absorbance readings.

Figure 4 : Biological Activity of BIOMAX™ purified rFC. The rFC was enriched by BIOMAX™-50 ultrafiltration and this protein sample was reacted with a range of absolute amounts of LPS (0.01-10 000 pg). Both 40 and 80 µg amounts of rFC showed enzymatic activity with 0.01 pg LPS.

Figure 5: Fluorimetric and colorimetric assays of LPS using purified rFC.

In both the fluorimetric and colorimetric assays, further purification of BIOMAX™-purified rFC by SEPHADEX™ G-100 dramatically improved the sensitivity of rFC to LPS. The conventional tube method of fluorimetric assay was compared with the
5 microcolorimetric assay for both the BIOMAX™ sample (B) and SEPHADEX™ G-100 purified sample (S). Amounts of rFC used were 40 µg and 100 µg for the fluorimetric and colorimetric assays, respectively.

Figure 6: Comparison of tube and plate fluorimetric assays. A comparison

of the fluorescence readings was made between the conventional tube method and the
10 microtiter plate method using 40 µg of the SEPHADEX™ G-100 purified rFC.

Figure 7: Colorimetric assay for LPS-induced Factor C enzyme activity of

rFC. A comparison is made of the amounts of culture supernatant proteins (60 and 100 µg) containing rFC, and the concentrations (2 and 4 mM) of the colorimetric substrate, Boc-Val-Pro-Arg-pNA. It is observed that as low as 0.01 pg endotoxin was optimally
15 detected with 100 µg culture supernatant at 2 mM pNA substrate.

Figure 8: Colorimetric assay of LPS using purified rFC. Similar to the

fluorimetric assay, the colorimetric test also showed that the SEPHADEX™ G-100 purified rFC exhibited improved sensitivity to LPS, where 40 µg of purified rFC (instead of 100 µg of BIOMAX™-purified rFC) was sufficient to detect subpicogram levels of
20 LPS.

Figure 9: Binding of rFC to lipid A assayed by BIACORE™ bioassay.

Binding between rFC produced from pFastBac/CrFC21 and immobilized lipid A (*E. coli* D31m4) was assayed using the BIACORE™ X biosensor (Pharmacia Biotech). BIOMAX™ and SEPHADEX™ G-100 purified supernatants of cultures of Sf9 cells
25 infected with wild-type baculovirus did not show any background binding to the

immobilized lipid A (plateau 1A). On the other hand, the rFC from the culture supernatant of Sf9 cells infected with pFastBac/CrFC21 specifically bound the immobilized lipid A with a net activity of 553 Response Units (plateau 2A). The protein samples, each at 1 mg/ml, were injected at 10 µl/min for 3 minutes over the
5 ligand monolayer that was previously immobilized on a HPA chip¹⁴.

Figure 10: rFC from Baculovirus binds LPS and lipid A from several Gram negative bacterial species. LPS from three different species of bacteria, *E coli*, *K. pneumoniae*, and *S. minnesota*, and lipid A from *S. minnesota*, were separated by electrophoresis and electroblotted onto an Immobilon™ PVDF membrane. Each
10 LPS/lipid A strip was incubated with rFC from pFastBac/CrFC21 (lane 1) or control culture supernatant from wild-type AcMNPV-infected Sf9 cells (lane 2). The results show that rFC binds LPS/lipid A from different species of Gram negative bacteria.

Figure 11: Microtiter plate-immobilized rFC for detection and removal of LPS. 10, 25 or 50 µg of partially-purified protein containing rFC derived from
15 baculoviral system (rFC Sf9) immobilized on a 96-well microtiter plate was capable of specifically recognizing and binding subpicogram levels of FITC-conjugated LPS. The efficacy of binding/detection of a range of LPS by various amounts of rFC protein immobilized onto the microtiter plate is shown.

Figure 12: Microtiter plate-immobilized rFC from various yeasts binds to LPS. Immobilized rFC derived from yeast (*P. pastoris*: rFC#8 {pHILD2/CrFC21} and rFCEE {pHILD2/CrFC21EE}; *S. cerevisiae*: YFC/6a {YepSec1/CrFC26Δ6a} and P21/26 {pEMBLyex4/CrFC21/26}). Native Factor C in *Carcinoscorpius* amoebocyte lysate, LAL (50 µg protein) were used as positive controls. There is
20 consistency in the efficiency of recognition of LPS-FITC and its binding to the immobilized rFC. The negative controls were w/tSf9 (wild-type Sf9 cells infected
25

with AcMNPV DNA alone) and rFCSN (rFC derived from a control yeast recombinant clone devoid of the LPS binding domain).

DETAILED DESCRIPTION OF THE INVENTION

The present application relates to rFC of the horseshoe crab.

5 A preferred horseshoe crab that can serve as a source of DNA or mRNA for producing the rFC of the invention is *Carcinoscorpius rotundicauda* (CrFC). The present invention relates especially to expression of rFC by means of baculovirus host-vector systems. The present application also relates to a fluorometric assay for endotoxin that makes use of the rFC expressed by recombinant DNA methods.

10 cDNAs encoding Factor C proteins from *Carcinoscorpius rotundicauda* have been previously described^{10,15}. rFC from *Carcinoscorpius rotundicauda* (rCrFC) has been produced *in vitro* by coupled transcription/translation systems^{10,15}. However, the present invention resides partly in the development of *in vivo* systems, especially using insect cells as the host cell, for efficient production of rFC by expression of cloned DNA.

15 Also, the protection of rFC from activation and subsequent self-proteolysis by binding of endotoxin which may be present in solutions used in isolation of the protein is described in reference 15. Basically, dimethylsulfoxide (Me₂SO, DMSO) is added to solutions which are used during the purification process. Even greater protection of the rFactor C is achieved by also adding an agent effective for chelating divalent metal ions
20 to the purification solutions.

cDNAs appropriate for expression in the presently-described system can be cDNAs encoding Factor C of any horseshoe crab. Two representative nucleotide sequences are presented as SEQ ID NO:1 and SEQ ID NO:3 (encoding the amino acid sequences of SEQ ID NOs:2 and 4). A composite DNA sequence, assembled from

incomplete cDNA fragments, encoding the Factor C of *Tachypleus tridentatus* is disclosed by Muta et al.¹

For use in the LPS binding assays and LPS removal according to the invention, the Factor C can be produced by any method typical in the art, but is preferably made in a eukaryotic host cell. Production of rFC in yeast host-vector systems is described in reference 16. As it has been the Inventors' recent experience that Factor C produced in yeast lacked serine protease activity, rFC for use in enzymatic activity-based assays is preferably produced by a baculovirus host-vector system.

"Stringent conditions" for hybridization are those that provide for hybridization of sequences having less than 15% mismatch, preferably less than 10% mismatch, most preferably 0% to 5% mismatch. Exemplary of such conditions, using probes of 50 bases or longer, are an aqueous solution of 0.9 M NaCl at 65 °C; an aqueous solution of 0.98 M NaCl, 20% formamide at 42-45 °C. The conditions will vary according to the length of the probe, its G+C content and other variables as known to the skilled practitioner¹⁷.

Exemplary wash conditions following hybridization are an aqueous solution of 0.9 M NaCl at 45-65 °C, preferably 55-65 °C. Lower salt, or addition of an organic solvent such as formamide, in the wash buffer will increase the stringency of the condition as known in the art.

A preferred hybridization condition is at 42°C in 50% formamide, 5x SSC, 1x Denhardt's solution, 20 mM phosphate buffer, pH 6.5, 50 µg/ml calf thymus DNA, 0.1% SDS. Salt and temperature conditions equivalent to the hybridization conditions employed can be calculated from the following equation¹⁸:

$$T_m = 81.5^{\circ}\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\% \text{G+C}) - 0.63(\% \text{formamide}) - (600/l),$$

where l = the length of the hybrid in base pairs.

A preferred washing condition is in 1x SSC, 0.1% SDS washing solution at room temperature, followed by washing at high stringency with 0.1x SSC, 0.1% SDS at 42°C and 2x with 0.1x SSC/0.1% SDS for 15 min. each at 42°C.

5 **Example 1: Recombinant constructs of CrFC cDNA in a baculovirus expression vector**

Plasmids and Sf9 cell culture

Sf9 insect cells were maintained as a monolayer culture in serum-free SF 900 II SFM medium supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin (Life Technologies, Inc.) in a humidified incubator (Forma, USA) at 27°C. The plasmid pFastBac ITM and the competent DH10Bac *E. coli* were from Life Technologies, Inc., USA.

Construction of pFastBac/CrFC21, transposition into E. coli and transfection into Sf9 insect cells.

The strategy for cloning CrFC21 into the pFastBac ITM (Life Technologies, Inc.) expression shuttle vector is shown in Fig 1. The recombinant plasmids were verified by restriction enzyme digestion. The 5' cloning sites were further confirmed by dideoxynucleotide sequencing using the forward primer designed from the -44 position of the polyhedrin promoter region, before they were used for transfection in insect cells. PCR and Southern analyses of the pFastBac/CrFC21 DNA confirmed the authenticity of recombinant baculoviruses.

The CrFC21 cDNA¹⁰ from pGEM11Zf+/CrFC21¹¹ was recloned in two steps into pBluescript II SK+ (pBSSK), to yield pBSSK/CrFC 21. Further manipulations using pBSSK/CrFC21 and the baculoviral expression vector, pFastBac ITM were carried out

using standard methods to clone full-length CrFC21, thus, yielding the recombinant construct, pFastBac/CrFC21 (Fig. 1). pFastBac/CrFC21 was transformed into competent *E. coli*, DH10Bac, and cultured in LB agar containing 50 µg/ml kanamycin, 7 µg/ml gentamycin, 10 µg/ml tetracycline, 30 µl of 2% X-gal and 40 µg/ml of IPTG. Screening¹⁹ for positive clones involved the use of the 2.3 kb ³²P-CrFC21/EE fragment as probe¹⁰. The recombinant bacmid DNA was isolated and transfected into Sf9 cells.

Example 2: Expression of rFC in insect host cells

Rapid microtiter-plate plaque assay

Early log phase recombinant Sf9 cells were seeded at 6.5×10^4 cells per well. The culture was incubated in a sealed bag at 27 °C for 1 h. Meanwhile, the virus stock was serially diluted 10-fold with SFM containing 10% FBS to give final dilutions of 10^{-2} to 10^{-4} . The BacPak™ Baculovirus Rapid Titer Kit (InVitrogen) was used for plaque assay. It is an immunoassay which uses a primary monoclonal antibody raised to an AcMNPV envelope glycoprotein (gp64). A secondary goat anti-mouse HRP-conjugated antibody enables visualization of the infected cells as blue-stained viral plaques or foci seen under the light microscope. The virus titer (pfu/ml) was calculated based on the following formula : (Average no. of foci per well x dilution factor x 40) x 2 where 40 represents the inoculum volume normalization factor.

Scale-up of infection of Sf9 cells for production of rFC

The culture supernatant from the 6-well plates was harvested and the viral stock was amplified by re-infection of Sf9 cells grown in 25 cm² flasks, using a multiplicity of infection (MOI) of 0.1 - 1.0. In such cultures, the viral stock reached a titer of 2×10^7 pfu/ml. Aliquots of this viral stock were re-inoculated at a MOI of 5-10

into Sf9 cells grown in 15 ml SFM medium in 75 cm² flasks. The volume of the viral inoculum was determined using the formula:

$$\frac{(\text{total no. of cells}) \times (\text{MOI in pfu/cell})}{(\text{viral titer in pfu/ml})}$$

5 Subsequently, Sf9 cells were passaged twice and conditioned to grow in suspension in 100 ml SFM medium, in spinner flasks (Bellco, USA). At the mid log phase of growth, the viral stock from the 75 cm² flask cells was inoculated at a MOI of 5-10. In the same manner, the cell culture volume was scaled up further in increasingly larger spinner flasks of 250, 500 and 1000 ml, infected with
10 proportionally increasing volumes of viral stock at the same MOI.

Preparation of protein samples from recombinant baculovirus-infected Sf9 cells.

(a) Cell lysate: Sf9 cells infected with the recombinant baculovirus at a MOI of 5-10 were harvested at 24, 48 and 72 h p.i. The cells were washed 3 times with pyrogen-free PBS and centrifuged at 3000 xg for 10 min at 4°C during each cycle of washing. The
15 cell pellet was resuspended in 2-3 volumes of PBS and subjected to 5 cycles of freeze-thawing at -80°C and 37°C, respectively. The cell debris was removed by centrifuging at 14000 xg for 10 min at 4°C. The supernatant containing the soluble protein fraction was stored at -20°C. This supernatant represents the cell lysate.

(b) Culture supernatant: At the respective times of harvest, the cell medium was
20 collected and centrifuged at 3000 xg for 10 min at 4°C to remove any cells or cell debris. The medium was then concentrated 10-fold by centrifugation through a BIOMAX™-50 kDa cutoff ultrafree membrane (Millipore) at 2000 xg for 20 min or more. The total proteins present in the cell lysate and culture supernatant were quantified by Bradford assay²⁰. Partial purification of rFC was carried out at 4 °C by gel filtration
25 chromatography through SEPHADEX™ G-100 (e.g. 1.5 x 90 cm), using 0.05 M Tris-

HCl (pH 7.5) containing 0.154 M NaCl. Fractions of 1 ml were collected and the void volume peak was concentrated. The protein concentration and Factor C enzyme activity were assayed for the resulting rFC. This preparation is henceforth referred to as "gel filtration-purified rFC".

5 *Western immunoblot detection of rFC*

Five µg of each cell lysate, or culture supernatant, harvested from 24, 48 and 72 h p.i. was analyzed on 10% SDS-PAGE gels, under denaturing conditions²¹. The electrophoretically-resolved bands were then transferred onto Immobilon™ PVDF membrane (Millipore, USA). The membrane was washed in PBS for 30 min, and
10 blocked in 1% skimmed milk-PBS for 1 h followed by overnight incubation with rabbit anti-Factor C antibody diluted 1:500 in 0.2% Tween-20-PBS containing 1% BSA. Horseradish peroxidase-conjugated secondary goat anti-rabbit antibody, diluted 1:10000, was subsequently incubated with the membrane. For visualization of protein bands, the membrane was treated with SUPERSIGNAL™ chemiluminescent
15 substrate (Pierce, USA) for 5-10 min, followed by 3 min exposure of the membrane to an X-ray film.

The Western analysis revealed 3 bands of immunoreactive rFC proteins of 132, 88 and 44 kDa, expressed by pFastBac/CrFC21 recombinant baculoviruses at 24, 48 and 72 h post infection, p.i. (Fig. 2A). At 24 h p.i., rFC was observed in the culture supernatant,
20 but not in the cell lysate. The 48 h and 72 h p.i. culture supernatant showed increasing amounts of rFC. The rFC in the cell lysate started to appear as a faint 132 kDa band only at 48 h p.i., and reached a substantial level at 72 h p.i. (Fig. 2B). The immunoblot thus showed that the bulk of the rFC produced was released from the infected Sf9 cells into the medium. This is probably due to lysis of the infected cells, which released the

recombinant protein. On ultracentrifugation at 100,000 xg for 1 h at 4 °C, rFC was found to be in the soluble fraction.

The results show that rFC protein was expressed correctly under the direction of the viral late promoter from the polyhedrin gene using the native translation start site from the CrFC cDNA. As there are six potential glycosylation sites in the CrFC cDNA sequence¹⁰; the protein band of 132 kDa represents the intact glycosylated form of Factor C. The 88 and 44 kDa proteins are likely the activated products of rFC whose molecular sizes correspond closely to the heavy and light chains, respectively, of double-chain Factor C³. Autoactivation could have occurred in the presence of picogram levels of ubiquitous endotoxin during the preparation of the protein sample for SDS-PAGE. Figure 2B shows a comparison of rFC from 72 h p.i., electrophoresed under reducing and non-reducing conditions of SDS-PAGE. The 88 and 44 kDa bands became more prominent under reducing conditions. Under non-reducing conditions, LPS-activated rFC still retained its 132 kDa band, thus indicating the double-chain form of rFC³. The presence of a double chain form of rFC was further proven when the BIOMAXTM-purified rFC was pre-incubated with LPS before Western blotting. Under reducing condition, the LPS-treated rFC showed activated products of 88 and 44 kDa which were absent in the untreated rFC sample (Fig. 2C). However, under non-reducing conditions (Fig. 2D), the 132 kDa band was intact for both the LPS-treated and -untreated rFC.

20 **Example 3: RFC binds lipid A, the biologically-potent component of LPS**

ELISA to determine lipid A binding by rFC

In order to visualize and test the ability of rFC to specifically bind the biologically potent component of LPS, diphosphoryl lipid A (*E. coli* K12, D31M4, List Biologicals, Inc., USA) ranging from 0.01 to 100 ng in 100 µl volumes was

drastically improved the specificity of lipid A binding (Fig. 3C). This assay indicates that rFC can be used for mass screening of pharmaceutical products for LPS contamination, with the capability of quantifying LPS. This efficacy is comparable to the commercially available natural lysate derived from the *Limulus* or *Tachypleus* amoebocyte lysate.

5 **Example 4: Immobilized rFC can be used to detect/remove LPS in a sample:**

Two hundred μ l samples containing either control wild-type supernatant (w/t Sf9, uninfected Sf9 cell supernatant) or partially-purified rFC samples (obtained by BIOMAX™-ultrafiltration), diluted in PBS to 10, 25 or 50- μ g total protein per 200 μ l were coated/immobilized onto each of the wells of a 96-well microtiter plate (NUNC, USA). The plates were left overnight at 4°C. Unbound protein was removed from the wells, and 200 μ l of 0.2% BSA (depyrogenized by ultrafiltration) dissolved in PBS was added to the wells for 1 h at 37°C, to block unoccupied sites. The wells were washed 3 times with PBS. This was followed by addition of 200 μ l FITC-conjugated LPS (*E. coli* 055:5B, List Biological Labs, USA) to the wells. The plate was incubated at 37°C for 1 h, after which each well was washed 6x with PBS. The fluorescence was read at Ex_{495nm} and Em_{525nm} using LS-50B Spectrofluorimeter (Perkin Elmer).

10

15

Wells coated with 10, 25 or 50 μ g of partially purified proteins containing rFC showed increasing efficiency of binding LPE-FITC. Blocking of the wells with 0.2% BSA reduced the background fluorescence reading, indicating improvement in the specificity of binding of LPS to the immobilized rFC. Immobilization of negative control proteins (w/tSf9: wild-type Sf9 cell culture supernatant of Sf9 cells infected with AcNMPV DNA alone, and rFCSN: yeast rFC derived from the truncated recombinant rFC devoid of LPS-binding domain described in ref. 16, to the wells did not capture or bind LPS, thus indicating the specificity of recognition of LPS by the immobilized rFC.

20

Example 5: The baculoviral rFC is enzymatically activated by LPS*Fluorimetric and colorimetric assays for LPS-activated rFC enzyme activity*

As a proenzyme, Factor C becomes catalytically activated by trace levels of LPS. Thus, conversion of its enzymatic substrate to product indicates the presence of LPS.

5 rFC samples present in the crude cell lysate and culture supernatant were used for analysis of LPS-activated Factor C enzyme activity by using two different substrates. The first, in a conventional tube assay format, is based on a modification of the fluorimetric assay of Iwanaga *et al.*²² Using rFC obtained from a 72 h p.i. culture supernatant, 10 µg total protein in a volume of 0.1 ml was mixed with 1.9 ml of 50
10 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl and 0.05 M CaCl₂. The mixtures were preincubated with 0.01 to 100 pg of LPS (*E. coli* 055:B5, Sigma) at 37 °C for 1 h before addition of 15 µl of 2 mM fluorimetric substrate, Boc-Val-Pro-Arg-MCA (Sigma). Incubation was continued for 30 min and the reaction was terminated with 0.1 ml glacial acetic acid. The product AMC was read in Fluorescence Units (FU) at
15 Ex_{380nm} (slit 10 nm) and Em_{460nm} (slit 5 nm) using a Perkin Elmer Luminescence Spectrophotometer (LS-50B). For multiple samples, this assay was routinely scaled down to 96-well microtiter plate assay. Briefly, the microassay involved 1 h pre-incubation of LPS with rFC in a volume of 100 µl, followed by addition of 1.5 µl of 2 mM fluorimetric substrate and 100 µl of 100 mM Tris-HCl, pH 8.0, containing 0.2 M
20 NaCl and 0.1 M CaCl₂ and further incubation for 30 min at 37 °C before termination of the reaction with 10 µl of glacial acetic acid. The fluorescence was read in a 96-well microtiter plate reader module.

The second enzymatic assay for LPS involved a modification of the colorimetric test²³ where preincubation of culture supernatant proteins with LPS
25 ranging from 0.01 to 10 pg was carried out at 37 °C for 1 h. The reaction volume was

scaled down to 200 μ l in 0.1 M Tris-HCl (pH 8.0) containing 5 mM $MgCl_2$. This was followed by addition of 50 μ l of 2 mM of a colorimetric substrate, Boc-Val-Pro-Arg-p-nitroanilide (Seikagaku, Japan). Incubation at 37 $^{\circ}C$ was resumed for 1 h before termination of the reaction with 28 μ l of glacial acetic acid. This substrate is
5 hydrolyzed by rFC to produce pNA that was measured colorimetrically at OD_{405nm} .

From 24 to 48 to 72 h p.i., there was progressively increasing trend in the enzymatic activity of rFC in supernatants of cultures of insect cells transformed with the construct of Example 1, as indicated by the increase in fluorimetric units of the AMC product hydrolyzed from Boc-Val-Pro-Arg-MCA substrate. A comparison of
10 the amount of total proteins present in the cell lysate (Lysate: 50 μ g) and culture supernatant (Sup: 5 μ g) illustrates that the culture supernatant from 72 h p.i. contained rFC that is >5-10 fold more effective in LPS detection. Twenty μ g of BIOMAXTM-purified rFC was able to detect 0.01 ng LPS. Using 40 to 80 μ g of this protein, the detection limit could be easily extended to LPS levels below 0.01 pg or 0.001 ng/ml
15 (Fig. 4). Purification of rFC by chromatography through SEPHADEXTM G-100 yielded enzymatic activity of even higher sensitivity to LPS (Fig. 5). It is envisaged that more elaborate purification of rFC following the methods covered in reference 24 would vastly improve the efficacy of the rFC for endotoxin detection. Furthermore, when the fluorimetric assay was modified to \sim 200 μ l, using a 96-well microtiter
20 plate, the sensitivity to LPS was improved by 10-fold (Fig. 6). This was directly attributable to the removal of background fluorescence by gel filtration.

Furthermore, the LPS-activated rFC enzyme assay was also conveniently quantifiable by a colorimetric assay with the Boc-Val-Pro-Arg-pNA substrate. The sensitivity to LPS was 0.1 pg (0.01 ng/ml) with 100 μ g of BIOMAXTM-50-treated

culture supernatant when 2 mM of the pNA substrate was employed (Fig. 7). Similar to the fluorimetric assay, the colorimetric test also showed that the SEPHADEX™ G-100-purified rFC exhibited improved sensitivity to LPS, where 40 µg of purified rFC (instead of 100 µg of BIOMAX™ rFC) was sufficient to detect subpicogram levels of LPS (Fig. 8). Use of gel filtration-purified rFC resulted in a 4-fold increase in sensitivity to LPS. A direct comparison of the 2 microassays revealed that with gel filtration-purified rFC, the colorimetric assay achieved sensitivity to LPS comparable to the fluorimetric assay. Thus, using the scaled down, yet improved sensitivity assay for LPS detection, high throughput screening of samples can be conveniently achieved by either the colorimetric or fluorimetric assay using the 96-well microtiter plate assays. This enables rapid and mass screening of samples with limited volumes.

The invention being thus described, modification of the invention with respect to various materials and methods will be apparent to one of ordinary skill in the art. Such modifications are to be considered as falling within the scope of the invention, which is defined by the claims hereinbelow.

References

Articles of the scientific and patent literature referred to herein are incorporated by reference in their entirety by citation thereto.

- 1 Muta, T., Miyata, T., Misumi, Y., Tokunaga, F., Nakamura, J., Toh, Y., Ikehara, Y., and Iwanaga, S. 1991. *J. Biol. Chem.* 266: 6554-6561.
- 2 Navas, M.M.A, Ding, J.L., and Ho, B. 1990. Inactivation of Factor C by dimethyl sulphoxide inhibits coagulation of the *Carcinoscorpius* amoebocyte lysate. *Biochem Mol Biol Int* 21:805-813.
- 3 Ding, J.L., Navas, M.M.A., and Ho, B. 1993. Two forms of Factor C from the amoebocytes of *Carcinoscorpius rotundicauda*: purification and characterization. *Biochim Biophys Acta* 1202:149-156.
- 4 Ho, B., Kim, J.C., and Ding, J.L. 1993. Electrophoretic analysis of endotoxin-activated gelation reaction of *Carcinoscorpius rotundicauda* amoebocyte lysate. *Biochem Mol Biol Int* 29:687-694.
- 5 Iwanaga, S. 1993. The limulus clotting reaction. *Current Opinion in Immunol* 5:74-82.
- 6 Ho, B. 1983. An improved *Limulus* gelation assay. *Microbios Lett* 24:81-84.
- 7 Cooper, J.F. 1975. Principles and applications of the limulus test for pyrogen in parenteral drugs. *Bull. Parent. Drug Ass.* 29: 122.
- 8 Novitsky, T. J. 1991. Discovery to commercialization: the blood of the horseshoe crab. *Oceanus* 27: 13-18.
- 9 Sekiguchi, K. and Nakamura, K. 1979. Ecology of the extant horseshoe crabs. In: *Biomedical Applications of the Horseshoe Crabs (Limulidae)*, Eds., Cohen *et al.*, Allan R. Liss, New York, pp. 37-49.

- 10 Ding, J.L., Navas III, M.A.A., and Ho, B. 1995. Molecular cloning and sequence analysis of Factor C cDNA from the Singapore horseshoe crab, *Carcinoscorpius rotundicauda*. *Mol Marine Biol Biotechnol* 4:90-103.
- 11 Roopashree, S.D., Chai, C., Ho, B, and Ding, J.L. 1995. Expression of *Carcinoscorpius rotundicauda* Factor C cDNA. *Biochem Mol Biol Intl* 4:841-849.
- 12 Roopashree, S.D., Ho, B, and Ding, J.L. 1996. Expression of *Carcinoscorpius rotundicauda* Factor C in *Pichia pastoris*. *Mol Marine Biol Biotechnol* 5: 334-343.
- 13 Ding, J.L., Chai, C., Pui, A.W.M. and Ho, B. 1997. Expression of full length and deletion homologues of *Carcinoscorpius rotundicauda* Factor C in *Saccharomyces cerevisiae*: immunoreactivity and endotoxin binding. *J Endotoxin Res.* 4(1): 33-43.
- 14 Pui, A.W.M., Ho, B. and Ding, J.L. 1998. Yeast recombinant Factor C from horseshoe crab binds endotoxin and causes bacteriostasis. *J. Endotoxin Research* (in press).
- 15 U.S. Patent 5,716,834.
- 16 Copending U.S. patent application Serial No. 08/596,405.
- 17 Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd. ed., c. 1989 by Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- 18 *ibid.* pp. 9.50-9.51.
- 19 Grunstein, M., and Hogness, D.S. 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc Natl Acad Sci* 72:3961.
- 20 Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72:248-254.

- 21 Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- 22 Iwanaga, S., Morita, T., Ohki, M. 1980. Endotoxin-sensitive substance. Japan Patent Agency Official Bulletin; S57-108018.
- 23 Nakamura, T., Morita, T., and Iwanaga, S. 1986 Lipopolysaccharide-sensitive serine protease zymogen (Factor C) found in *Limulus* hemocytes: Isolation and characterization. *Eur J Biochem* 154: 511-521.
- 24 U.S. Patent 5,712,144.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(ii) TITLE OF INVENTION: Cloning and Expression of *Carcinoscorpius rotundicauda* Factor C in a Baculoviral Host-vector System

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Birch, Stewart, Kolasch & Birch
(B) STREET: 8110 Gatehouse Road, Suite 500 East
(C) CITY: Falls Church
(D) STATE: Virginia
(E) COUNTRY: USA
(F) ZIP: 22042

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Murphy, Jr., Gerald M.
(B) REGISTRATION NUMBER: 28,977
(C) REFERENCE/DOCKET NUMBER: 1781-105P

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (703) 205-8000
(B) TELEFAX: (703) 205-8050
(C) TELEX: 248345

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4182 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Carcinoscorpius rotundicauda*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 569..3817

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTATTTAATG TCTCAACGGT AAAGGTTTCA TTGTAGCTAA TATTTAACTT CCTCCCTGTG	60
CCCCAAATCG CGAGTATGAC GTCAGTTAAG ACTTCGTATT TTAAGAGTTA AACACGAGCC	120
TAAAGAGCG ATATTTTTTT TGTAAACAC TTCCAACCTA ATACAATTGG CAAACTTTCA	180
AAAATAAAGT GGAAAAGGAG GTAAAAAAGA TGAAAAAAT TCGCATACAA TAGAATACAA	240
TAAATGTGT TGTCTTTACT GTCAACACTT ACTGTTCTGTT CGGTCACAGC TGTGAATCGG	300
GGTGACTTTA TGTTTGTAGT GGTCTTAAAA ACGGGTACTT GGTGTGTTTG AAAATTTTAA	360
AACCTACATA TGATTCTCCT AAAATTTTGT TTATAATTA GCACCATTG CGACCTAAT	420
CTTTTTTGTA GTCTTAAGTT TAGTTGACAT AAAACAAAA TTTGTAACAA CACACGGTAT	480
AAACTAAATA GCTTCAGATG GGTCGTATGA CAAGGAACT TTAAATAAT TATGAAAGTT	540
TTTTTAAAT TTGACTAAGG TTTAGATT ATG TGG GTG ACA TGC TTC GAC ACG	592
Met Trp Val Thr Cys Phe Asp Thr	
1 5	
TTT CTT TTT GTT TGT GAA AGT TCA GTT TTC TGT TTG TTG TGT GTG TGG	640
Phe Leu Phe Val Cys Glu Ser Ser Val Phe Cys Leu Leu Cys Val Trp	
10 15 20	
AGG TTT GGT TTC TGT AGG TGG CGT GTT TTC TAC AGT TTT CCA TTC GTT	688
Arg Phe Gly Phe Cys Arg Trp Arg Val Phe Tyr Ser Phe Pro Phe Val	
25 30 35 40	
AAG TCA ACA GTT GTT TTA TTA CAG TGT TAC CAT TAC TCT CTC CAC AAT	736
Lys Ser Thr Val Val Leu Leu Gln Cys Tyr His Tyr Ser Leu His Asn	
45 50 55	
ACC TCA AAG TTC TAC TCT GTG AAT CCT GAC AAG CCA GAG TAC ATT CTT	784
Thr Ser Lys Phe Tyr Ser Val Asn Pro Asp Lys Pro Glu Tyr Ile Leu	
60 65 70	
TCA GGT TTA GTT CTA GGG CTA CTA GCC CAA AAA ATG CGC CCA GTT CAG	832
Ser Gly Leu Val Leu Gly Leu Leu Ala Gln Lys Met Arg Pro Val Gln	
75 80 85	
TCC AAA GGA GTA GAT CTA GGC TTG TGT GAT GAA ACG AGG TTC GAG TGT	880
Ser Lys Gly Val Asp Leu Gly Leu Cys Asp Glu Thr Arg Phe Glu Cys	
90 95 100	
AAG TGT GGC GAT CCA GGC TAT GTG TTC AAC ATT CCA GTG AAA CAA TGT	928
Lys Cys Gly Asp Pro Gly Tyr Val Phe Asn Ile Pro Val Lys Gln Cys	
105 110 115 120	
ACA TAC TTT TAT CGA TGG AGG CCG TAT TGT AAA CCA TGT GAT GAC CTG	976
Thr Tyr Phe Tyr Arg Trp Arg Pro Tyr Cys Lys Pro Cys Asp Asp Leu	
125 130 135	

27

GAG GCT AAG GAT ATT TGT CCA AAG TAC AAA CGA TGT CAA GAG TGT AAG Glu Ala Lys Asp Ile Cys Pro Lys Tyr Lys Arg Cys Gln Glu Cys Lys 140 145 150	1024
GCT GGT CTT GAT AGT TGT GTT ACT TGT CCA CCT AAC AAA TAT GGT ACT Ala Gly Leu Asp Ser Cys Val Thr Cys Pro Pro Asn Lys Tyr Gly Thr 155 160 165	1072
TGG TGT AGC GGT GAA TGT CAG TGT AAG AAT GGA GGT ATC TGT GAC CAG Trp Cys Ser Gly Glu Cys Gln Cys Lys Asn Gly Gly Ile Cys Asp Gln 170 175 180	1120
AGG ACA GGA GCT TGT GCA TGT CGT GAC AGA TAT GAA GGG GTG CAC TGT Arg Thr Gly Ala Cys Ala Cys Arg Asp Arg Tyr Glu Gly Val His Cys 185 190 195 200	1168
GAA ATT CTC AAA GGT TGT CCT CTT CTT CCA TCG GAT TCT CAG GTT CAG Glu Ile Leu Lys Gly Cys Pro Leu Leu Pro Ser Asp Ser Gln Val Gln 205 210 215	1216
GAA GTC AGA AAT CCA CCA GAT AAT CCC CAA ACT ATT GAC TAC AGC TGT Glu Val Arg Asn Pro Pro Asp Asn Pro Gln Thr Ile Asp Tyr Ser Cys 220 225 230	1264
TCA CCA GGG TTC AAG CTT AAG GGT ATG GCA CGA ATT AGC TGT CTC CCA Ser Pro Gly Phe Lys Leu Lys Gly Met Ala Arg Ile Ser Cys Leu Pro 235 240 245	1312
AAT GGA CAG TGG AGT AAC TTT CCA CCC AAA TGT ATT CGA GAA TGT GCC Asn Gly Gln Trp Ser Asn Phe Pro Pro Lys Cys Ile Arg Glu Cys Ala 250 255 260	1360
ATG GTT TCA TCT CCA GAA CAT GGG AAA GTG AAT GCT CTT AGT GGT GAT Met Val Ser Ser Pro Glu His Gly Lys Val Asn Ala Leu Ser Gly Asp 265 270 275 280	1408
ATG ATA GAA GGG GCT ACT TTA CGG TTC TCA TGT GAT AGT CCC TAC TAC Met Ile Glu Gly Ala Thr Leu Arg Phe Ser Cys Asp Ser Pro Tyr Tyr 285 290 295	1456
TTG ATT GGT CAA GAA ACA TTA ACC TGT CAG GGT AAT GGT CAG TGG AAT Leu Ile Gly Gln Glu Thr Leu Thr Cys Gln Gly Asn Gly Gln Trp Asn 300 305 310	1504
GGA CAG ATA CCA CAA TGT AAG AAC TTA GTC TTC TGT CCT GAC CTG GAT Gly Gln Ile Pro Gln Cys Lys Asn Leu Val Phe Cys Pro Asp Leu Asp 315 320 325	1552
CCT GTA AAC CAT GCT GAA CAC AAG GTT AAA ATT GGT GTG GAA CAA AAA Pro Val Asn His Ala Glu His Lys Val Lys Ile Gly Val Glu Gln Lys 330 335 340	1600
TAT GGT CAG TTT CCT CAA GGC ACT GAA GTG ACC TAT ACG TGT TCG GGT Tyr Gly Gln Phe Pro Gln Gly Thr Glu Val Thr Tyr Thr Cys Ser Gly 345 350 355 360	1648
AAC TAC TTC TTG ATG GGT TTT GAC ACC TTA AAA TGT AAC CCT GAT GGG Asn Tyr Phe Leu Met Gly Phe Asp Thr Leu Lys Cys Asn Pro Asp Gly 365 370 375	1696

TCT TGG TCA GGA TCA CAG CCA TCC TGT GTT AAA GTG GCA GAC AGA GAG Ser Trp Ser Gly Ser Gln Pro Ser Cys Val Lys Val Ala Asp Arg Glu 380 385 390	1744
GTC GAC TGT GAC AGT AAA GCT GTA GAC TTC TTG GAT GAT GTT GGT GAA Val Asp Cys Asp Ser Lys Ala Val Asp Phe Leu Asp Asp Val Gly Glu 395 400 405	1792
CCT GTC AGG ATC CAC TGT CCT GCT GGC TGT TCT TTG ACA GCT GGT ACT Pro Val Arg Ile His Cys Pro Ala Gly Cys Ser Leu Thr Ala Gly Thr 410 415 420	1840
GTG TGG GGT ACA GCC ATA TAC CAT GAA CTT TCC TCA GTG TGT CGT GCA Val Trp Gly Thr Ala Ile Tyr His Glu Leu Ser Ser Val Cys Arg Ala 425 430 435 440	1888
GCC ATC CAT GCT GGC AAG CTT CCA AAC TCT GGA GGA GCG GTG CAT GTT Ala Ile His Ala Gly Lys Leu Pro Asn Ser Gly Gly Ala Val His Val 445 450 455	1936
GTG AAC AAT GGC CCC TAC TCG GAC TTT CTG GGT AGT GAC CTG AAT GGG Val Asn Asn Gly Pro Tyr Ser Asp Phe Leu Gly Ser Asp Leu Asn Gly 460 465 470	1984
ATA AAA TCC GAA GAG TTG AAG TCT CTT GCC CGG AGT TTC CGA TTC GAT Ile Lys Ser Glu Glu Leu Lys Ser Leu Ala Arg Ser Phe Arg Phe Asp 475 480 485	2032
TAT GTC AGT TCC TCC ACA GCA GGT AAA TCA GGA TGT CCT GAT GGA TGG Tyr Val Ser Ser Ser Thr Ala Gly Lys Ser Gly Cys Pro Asp Gly Trp 490 495 500	2080
TTT GAG GTA GAC GAG AAC TGT GTG TAC GTT ACA TCA AAA CAG AGA GCC Phe Glu Val Asp Glu Asn Cys Val Tyr Val Thr Ser Lys Gln Arg Ala 505 510 515 520	2128
TGG GAA AGA GCT CAA GGT GTG TGT ACC AAT ATG GCT GCT CGT CTT GCT Trp Glu Arg Ala Gln Gly Val Cys Thr Asn Met Ala Ala Arg Leu Ala 525 530 535	2176
GTG CTG GAC AAA GAT GTA ATT CCA AAT TCA TTG ACT GAG ACT CTA CGA Val Leu Asp Lys Asp Val Ile Pro Asn Ser Leu Thr Glu Thr Leu Arg 540 545 550	2224
GGG AAA GGG TTA ACA ACC ACG TGG ATA GGA TTG CAC AGA CTA GAT GCT Gly Lys Gly Leu Thr Thr Thr Trp Ile Gly Leu His Arg Leu Asp Ala 555 560 565	2272
GAG AAG CCC TTT ATT TGG GAG TTA ATG GAT CGT AGT AAT GTG GTT CTG Glu Lys Pro Phe Ile Trp Glu Leu Met Asp Arg Ser Asn Val Val Leu 570 575 580	2320
AAT GAT AAC CTA ACA TTC TGG GCC TCT GGC GAA CCT GGA AAT GAA ACT Asn Asp Asn Leu Thr Phe Trp Ala Ser Gly Glu Pro Gly Asn Glu Thr 585 590 595 600	2368
AAC TGT GTA TAT ATG GAC ATC CAA GAT CAG TTG CAG TCT GTG TGG AAA Asn Cys Val Tyr Met Asp Ile Gln Asp Gln Leu Gln Ser Val Trp Lys 605 610 615	2416

29

ACC AAG TCA TGT TTT CAG CCC TCA AGT TTT GCT TGC ATG ATG GAT CTG Thr Lys Ser Cys Phe Gln Pro Ser Ser Phe Ala Cys Met Met Asp Leu 620 625 630	2464
TCA GAC AGA AAT AAA GCC AAA TGC GAT GAT CCT GGA TCA CTG GAA AAT Ser Asp Arg Asn Lys Ala Lys Cys Asp Asp Pro Gly Ser Leu Glu Asn 635 640 645	2512
GGA CAC GCC ACA CTT CAT GGA CAA AGT ATT GAT GGG TTC TAT GCT GGT Gly His Ala Thr Leu His Gly Gln Ser Ile Asp Gly Phe Tyr Ala Gly 650 655 660	2560
TCT TCT ATA AGG TAC AGC TGT GAG GTT CTC CAC TAC CTC AGT GGA ACT Ser Ser Ile Arg Tyr Ser Cys Glu Val Leu His Tyr Leu Ser Gly Thr 665 670 675 680	2608
GAA ACC GTA ACT TGT ACA ACA AAT GGC ACA TGG AGT GCT CCT AAA CCT Glu Thr Val Thr Cys Thr Thr Asn Gly Thr Trp Ser Ala Pro Lys Pro 685 690 695	2656
CGA TGT ATC AAA GTC ATC ACC TGC CAA AAC CCC CCT GTA CCA TCA TAT Arg Cys Ile Lys Val Ile Thr Cys Gln Asn Pro Pro Val Pro Ser Tyr 700 705 710	2704
GGT TCT GTG GAA ATC AAA CCC CCA AGT CGG ACA AAC TCG ATA AGT CGT Gly Ser Val Glu Ile Lys Pro Pro Ser Arg Thr Asn Ser Ile Ser Arg 715 720 725	2752
GTT GGG TCA CCT TTC TTG AGG TTG CCA CGG TTA CCC CTC CCA TTA GCC Val Gly Ser Pro Phe Leu Arg Leu Pro Arg Leu Pro Leu Pro Leu Ala 730 735 740	2800
AGA GCA GCC AAA CCT CCT CCA AAA CCT AGA TCC TCA CAA CCC TCT ACT Arg Ala Ala Lys Pro Pro Pro Lys Pro Arg Ser Ser Gln Pro Ser Thr 745 750 755 760	2848
GTG GAC TTG GCT TCT AAA GTT AAA CTA CCT GAA GGT CAT TAC CGG GTA Val Asp Leu Ala Ser Lys Val Lys Leu Pro Glu Gly His Tyr Arg Val 765 770 775	2896
GGG TCT CGA GCC ATT TAC ACG TGC GAG TCG AGA TAC TAC GAA CTA CTT Gly Ser Arg Ala Ile Tyr Thr Cys Glu Ser Arg Tyr Tyr Glu Leu Leu 780 785 790	2944
GGA TCT CAA GGC AGA AGA TGT GAC TCT AAT GGA AAC TGG AGT GGT CGG Gly Ser Gln Gly Arg Arg Cys Asp Ser Asn Gly Asn Trp Ser Gly Arg 795 800 805	2992
CCA GCG AGC TGT ATT CCA GTT TGT GGA CGG TCA GAC TCT CCT CGT TCT Pro Ala Ser Cys Ile Pro Val Cys Gly Arg Ser Asp Ser Pro Arg Ser 810 815 820	3040
CCT TTT ATC TGG AAT GGG AAT TCT ACA GAA ATA GGT CAG TGG CCG TGG Pro Phe Ile Trp Asn Gly Asn Ser Thr Glu Ile Gly Gln Trp Pro Trp 825 830 835 840	3088
CAG GCA GGA ATC TCT AGA TGG CTT GCA GAC CAC AAT ATG TGG TTT CTC Gln Ala Gly Ile Ser Arg Trp Leu Ala Asp His Asn Met Trp Phe Leu 845 850 855	3136

CAG TGT GGA GGA TCT CTA TTG AAT GAG AAA TGG ATC GTC ACT GCT GCC Gln Cys Gly Gly Ser Leu Leu Asn Glu Lys Trp Ile Val Thr Ala Ala 860 865 870	3184
CAC TGT GTC ACC TAC TCT GCT ACT GCT GAG ATT ATT GAC CCC AAT CAG His Cys Val Thr Tyr Ser Ala Thr Ala Glu Ile Ile Asp Pro Asn Gln 875 880 885	3232
TTT AAA ATG TAT CTG GGC AAG TAC TAC CGT GAT GAC AGT AGA GAC GAT Phe Lys Met Tyr Leu Gly Lys Tyr Tyr Arg Asp Asp Ser Arg Asp Asp 890 895 900	3280
GAC TAT GTA CAA GTA AGA GAG GCT CTT GAG ATC CAC GTG AAT CCT AAC Asp Tyr Val Gln Val Arg Glu Ala Leu Glu Ile His Val Asn Pro Asn 905 910 915 920	3328
TAC GAC CCC GGC AAT CTC AAC TTT GAC ATA GCC CTA ATT CAA CTG AAA Tyr Asp Pro Gly Asn Leu Asn Phe Asp Ile Ala Leu Ile Gln Leu Lys 925 930 935	3376
ACT CCT GTT ACT TTG ACA ACA CGA GTC CAA CCA ATC TGT CTG CCT ACT Thr Pro Val Thr Leu Thr Thr Arg Val Gln Pro Ile Cys Leu Pro Thr 940 945 950	3424
GAC ATC ACA ACA AGA GAA CAC TTG AAG GAG GGA ACA TTA GCA GTG GTG Asp Ile Thr Thr Arg Glu His Leu Lys Glu Gly Thr Leu Ala Val Val 955 960 965	3472
ACA GGT TGG GGT TTG AAT GAA AAC AAC ACC TAT TCA GAG ACG ATT CAA Thr Gly Trp Gly Leu Asn Glu Asn Asn Thr Tyr Ser Glu Thr Ile Gln 970 975 980	3520
CAA GCT GTG CTA CCT GTT GTT GCA GCC AGC ACC TGT GAA GAG GGG TAC Gln Ala Val Leu Pro Val Val Ala Ala Ser Thr Cys Glu Glu Gly Tyr 985 990 995 1000	3568
AAG GAA GCA GAC TTA CCA CTG ACA GTA ACA GAG AAC ATG TTC TGT GCA Lys Glu Ala Asp Leu Pro Leu Thr Val Thr Glu Asn Met Phe Cys Ala 1005 1010 1015	3616
GGT TAC AAG AAG GGA CGT TAT GAT GCC TGC AGT GGG GAC AGT GGA GGA Gly Tyr Lys Lys Gly Arg Tyr Asp Ala Cys Ser Gly Asp Ser Gly Gly 1020 1025 1030	3664
CCT TTA GTG TTT GCT GAT GAT TCC CGT ACC GAA AGG CGG TGG GTC TTG Pro Leu Val Phe Ala Asp Asp Ser Arg Thr Glu Arg Arg Trp Val Leu 1035 1040 1045	3712
GAA GGG ATT GTC AGC TGG GGC AGT CCC AGT GGA TGT GGC AAG GCG AAC Glu Gly Ile Val Ser Trp Gly Ser Pro Ser Gly Cys Gly Lys Ala Asn 1050 1055 1060	3760
CAG TAC GGG GGC TTC ACT AAA GTT AAC GTT TTC CTG TCA TGG ATT AGG Gln Tyr Gly Gly Phe Thr Lys Val Asn Val Phe Leu Ser Trp Ile Arg 1065 1070 1075 1080	3808
CAG TTC ATT TGAACTGAT CTAAATATTT TAAGCATGGT TATAAACGTC Gln Phe Ile	3857
TTGTTTCCTAT TATTGCTTTA CTGGTTTAAAC CCATAAGAAG GTTAACGGGG TAAGGCACAA	3917

```

GGATCATTGT TTCTGTTTGT TTTTACAAAT GGTTCCTTTA GTCAGTGAAT GAGAATAGTA 3977
TCCATTGGAG ACTGTTACCT TTTATTCTAC CTTTTTATAT TACTATGCAA GTATTTGGGA 4037
TATCTTCTAC ACATGAAAAT TCTGTCATTT TACCATAAAT TTGGTTTCTG GTGTGTGTGT 4097
TAAGTCCACC ACTAGAGAAC GATGTAATTT TCAATAGTAC ATGAAATAAA TATAGAACAA 4157
ATCTATTATA AAAAAAAAAA AAAAA 4182

```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1083 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Trp Val Thr Cys Phe Asp Thr Phe Leu Phe Val Cys Glu Ser Ser
 1             5             10             15
Val Phe Cys Leu Leu Cys Val Trp Arg Phe Gly Phe Cys Arg Trp Arg
      20             25             30
Val Phe Tyr Ser Phe Pro Phe Val Lys Ser Thr Val Val Leu Leu Gln
      35             40             45
Cys Tyr His Tyr Ser Leu His Asn Thr Ser Lys Phe Tyr Ser Val Asn
      50             55             60
Pro Asp Lys Pro Glu Tyr Ile Leu Ser Gly Leu Val Leu Gly Leu Leu
      65             70             75             80
Ala Gln Lys Met Arg Pro Val Gln Ser Lys Gly Val Asp Leu Gly Leu
      85             90             95
Cys Asp Glu Thr Arg Phe Glu Cys Lys Cys Gly Asp Pro Gly Tyr Val
      100             105             110
Phe Asn Ile Pro Val Lys Gln Cys Thr Tyr Phe Tyr Arg Trp Arg Pro
      115             120             125
Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys Asp Ile Cys Pro Lys
      130             135             140
Tyr Lys Arg Cys Gln Glu Cys Lys Ala Gly Leu Asp Ser Cys Val Thr
      145             150             155             160
Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser Gly Glu Cys Gln Cys
      165             170             175
Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly Ala Cys Ala Cys Arg
      180             185             190

```


Asp Arg Tyr Glu Gly Val His Cys Glu Ile Leu Lys Gly Cys Pro Leu
 195 200 205
 Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg Asn Pro Pro Asp Asn
 210 215 220
 Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly
 225 230 235 240
 Met Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln Trp Ser Asn Phe Pro
 245 250 255
 Pro Lys Cys Ile Arg Glu Cys Ala Met Val Ser Ser Pro Glu His Gly
 260 265 270
 Lys Val Asn Ala Leu Ser Gly Asp Met Ile Glu Gly Ala Thr Leu Arg
 275 280 285
 Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly Gln Glu Thr Leu Thr
 290 295 300
 Cys Gln Gly Asn Gly Gln Trp Asn Gly Gln Ile Pro Gln Cys Lys Asn
 305 310 315 320
 Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn His Ala Glu His Lys
 325 330 335
 Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln Phe Pro Gln Gly Thr
 340 345 350
 Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe Leu Met Gly Phe Asp
 355 360 365
 Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser Gly Ser Gln Pro Ser
 370 375 380
 Cys Val Lys Val Ala Asp Arg Glu Val Asp Cys Asp Ser Lys Ala Val
 385 390 395 400
 Asp Phe Leu Asp Asp Val Gly Glu Pro Val Arg Ile His Cys Pro Ala
 405 410 415
 Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly Thr Ala Ile Tyr His
 420 425 430
 Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His Ala Gly Lys Leu Pro
 435 440 445
 Asn Ser Gly Gly Ala Val His Val Val Asn Asn Gly Pro Tyr Ser Asp
 450 455 460
 Phe Leu Gly Ser Asp Leu Asn Gly Ile Lys Ser Glu Glu Leu Lys Ser
 465 470 475 480
 Leu Ala Arg Ser Phe Arg Phe Asp Tyr Val Ser Ser Ser Thr Ala Gly
 485 490 495
 Lys Ser Gly Cys Pro Asp Gly Trp Phe Glu Val Asp Glu Asn Cys Val
 500 505 510

Tyr Val Thr Ser Lys Gln Arg Ala Trp Glu Arg Ala Gln Gly Val Cys
 515 520 525
 Thr Asn Met Ala Ala Arg Leu Ala Val Leu Asp Lys Asp Val Ile Pro
 530 535 540
 Asn Ser Leu Thr Glu Thr Leu Arg Gly Lys Gly Leu Thr Thr Thr Trp
 545 550 555 560
 Ile Gly Leu His Arg Leu Asp Ala Glu Lys Pro Phe Ile Trp Glu Leu
 565 570 575
 Met Asp Arg Ser Asn Val Val Leu Asn Asp Asn Leu Thr Phe Trp Ala
 580 585 590
 Ser Gly Glu Pro Gly Asn Glu Thr Asn Cys Val Tyr Met Asp Ile Gln
 595 600 605
 Asp Gln Leu Gln Ser Val Trp Lys Thr Lys Ser Cys Phe Gln Pro Ser
 610 615 620
 Ser Phe Ala Cys Met Met Asp Leu Ser Asp Arg Asn Lys Ala Lys Cys
 625 630 635 640
 Asp Asp Pro Gly Ser Leu Glu Asn Gly His Ala Thr Leu His Gly Gln
 645 650 655
 Ser Ile Asp Gly Phe Tyr Ala Gly Ser Ser Ile Arg Tyr Ser Cys Glu
 660 665 670
 Val Leu His Tyr Leu Ser Gly Thr Glu Thr Val Thr Cys Thr Thr Asn
 675 680 685
 Gly Thr Trp Ser Ala Pro Lys Pro Arg Cys Ile Lys Val Ile Thr Cys
 690 695 700
 Gln Asn Pro Pro Val Pro Ser Tyr Gly Ser Val Glu Ile Lys Pro Pro
 705 710 715 720
 Ser Arg Thr Asn Ser Ile Ser Arg Val Gly Ser Pro Phe Leu Arg Leu
 725 730 735
 Pro Arg Leu Pro Leu Pro Leu Ala Arg Ala Ala Lys Pro Pro Pro Lys
 740 745 750
 Pro Arg Ser Ser Gln Pro Ser Thr Val Asp Leu Ala Ser Lys Val Lys
 755 760 765
 Leu Pro Glu Gly His Tyr Arg Val Gly Ser Arg Ala Ile Tyr Thr Cys
 770 775 780
 Glu Ser Arg Tyr Tyr Glu Leu Leu Gly Ser Gln Gly Arg Arg Cys Asp
 785 790 795 800
 Ser Asn Gly Asn Trp Ser Gly Arg Pro Ala Ser Cys Ile Pro Val Cys
 805 810 815
 Gly Arg Ser Asp Ser Pro Arg Ser Pro Phe Ile Trp Asn Gly Asn Ser
 820 825 830

Thr Glu Ile Gly Gln Trp Pro Trp Gln Ala Gly Ile Ser Arg Trp Leu
 835 840 845
 Ala Asp His Asn Met Trp Phe Leu Gln Cys Gly Gly Ser Leu Leu Asn
 850 855 860
 Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Thr Tyr Ser Ala Thr
 865 870 875 880
 Ala Glu Ile Ile Asp Pro Asn Gln Phe Lys Met Tyr Leu Gly Lys Tyr
 885 890 895
 Tyr Arg Asp Asp Ser Arg Asp Asp Asp Tyr Val Gln Val Arg Glu Ala
 900 905 910
 Leu Glu Ile His Val Asn Pro Asn Tyr Asp Pro Gly Asn Leu Asn Phe
 915 920 925
 Asp Ile Ala Leu Ile Gln Leu Lys Thr Pro Val Thr Leu Thr Thr Arg
 930 935 940
 Val Gln Pro Ile Cys Leu Pro Thr Asp Ile Thr Thr Arg Glu His Leu
 945 950 955 960
 Lys Glu Gly Thr Leu Ala Val Val Thr Gly Trp Gly Leu Asn Glu Asn
 965 970 975
 Asn Thr Tyr Ser Glu Thr Ile Gln Gln Ala Val Leu Pro Val Val Ala
 980 985 990
 Ala Ser Thr Cys Glu Glu Gly Tyr Lys Glu Ala Asp Leu Pro Leu Thr
 995 1000 1005
 Val Thr Glu Asn Met Phe Cys Ala Gly Tyr Lys Lys Gly Arg Tyr Asp
 1010 1015 1020
 Ala Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Phe Ala Asp Asp Ser
 1025 1030 1035 1040
 Arg Thr Glu Arg Arg Trp Val Leu Glu Gly Ile Val Ser Trp Gly Ser
 1045 1050 1055
 Pro Ser Gly Cys Gly Lys Ala Asn Gln Tyr Gly Gly Phe Thr Lys Val
 1060 1065 1070
 Asn Val Phe Leu Ser Trp Ile Arg Gln Phe Ile
 1075 1080

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3448 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Carcinoscorpius rotundicauda*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 18..3074

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTGAAGGTAA CTTAAGT ATG GTC TTA GCG TCG TTT TTG GTG TCT GGT TTA	50
Met Val Leu Ala Ser Phe Leu Val Ser Gly Leu	
1 5 10	
GTT CTA GGG CTA CTA GCC CAA AAA ATG CGC CCA GTT CAG TCC AAA GGA	98
Val Leu Gly Leu Leu Ala Gln Lys Met Arg Pro Val Gln Ser Lys Gly	
15 20 25	
GTA GAT CTA GGC TTG TGT GAT GAA ACG AGG TTC GAG TGT AAG TGT GGC	146
Val Asp Leu Gly Leu Cys Asp Glu Thr Arg Phe Glu Cys Lys Cys Gly	
30 35 40	
GAT CCA GGC TAT GTG TTC AAC ATT CCA GTG AAA CAA TGT ACA TAC TTT	194
Asp Pro Gly Tyr Val Phe Asn Ile Pro Val Lys Gln Cys Thr Tyr Phe	
45 50 55	
TAT CGA TGG AGG CCG TAT TGT AAA CCA TGT GAT GAC CTG GAG GCT AAG	242
Tyr Arg Trp Arg Pro Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys	
60 65 70 75	
GAT ATT TGT CCA AAG TAC AAA CGA TGT CAA GAG TGT AAG GCT GGT CTT	290
Asp Ile Cys Pro Lys Tyr Lys Arg Cys Gln Glu Cys Lys Ala Gly Leu	
80 85 90	
GAT AGT TGT GTT ACT TGT CCA CCT AAC AAA TAT GGT ACT TGG TGT AGC	338
Asp Ser Cys Val Thr Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser	
95 100 105	
GGT GAA TGT CAG TGT AAG AAT GGA GGT ATC TGT GAC CAG AGG ACA GGA	386
Gly Glu Cys Gln Cys Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly	
110 115 120	
GCT TGT GCA TGT CGT GAC AGA TAT GAA GGG GTG CAC TGT GAA ATT CTC	434
Ala Cys Ala Cys Arg Asp Arg Tyr Glu Gly Val His Cys Glu Ile Leu	
125 130 135	
AAA GGT TGT CCT CTT CTT CCA TCG GAT TCT CAG GTT CAG GAA GTC AGA	482
Lys Gly Cys Pro Leu Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg	
140 145 150 155	
AAT CCA CCA GAT AAT CCC CAA ACT ATT GAC TAC AGC TGT TCA CCA GGG	530
Asn Pro Pro Asp Asn Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly	
160 165 170	
TTC AAG CTT AAG GGT ATG GCA CGA ATT AGC TGT CTC CCA AAT GGA CAG	578
Phe Lys Leu Lys Gly Met Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln	
175 180 185	

TGG AGT AAC TTT CCA CCC AAA TGT ATT CGA GAA TGT GCC ATG GTT TCA Trp Ser Asn Phe Pro Pro Lys Cys Ile Arg Glu Cys Ala Met Val Ser 190 195 200	626
TCT CCA GAA CAT GGG AAA GTG AAT GCT CTT AGT GGT GAT ATG ATA GAA Ser Pro Glu His Gly Lys Val Asn Ala Leu Ser Gly Asp Met Ile Glu 205 210 215	674
GGG GCT ACT TTA CGG TTC TCA TGT GAT AGT CCC TAC TAC TTG ATT GGT Gly Ala Thr Leu Arg Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly 220 225 230 235	722
CAA GAA ACA TTA ACC TGT CAG GGT AAT GGT CAG TGG AAT GGA CAG ATA Gln Glu Thr Leu Thr Cys Gln Gly Asn Gly Gln Trp Asn Gly Gln Ile 240 245 250	770
CCA CAA TGT AAG AAC TTG GTC TTC TGT CCT GAC CTG GAT CCT GTA AAC Pro Gln Cys Lys Asn Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn 255 260 265	818
CAT GCT GAA CAC AAG GTT AAA ATT GGT GTG GAA CAA AAA TAT GGT CAG His Ala Glu His Lys Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln 270 275 280	866
TTT CCT CAA GGC ACT GAA GTG ACC TAT ACG TGT TCG GGT AAC TAC TTC Phe Pro Gln Gly Thr Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe 285 290 295	914
TTG ATG GGT TTT GAC ACC TTA AAA TGT AAC CCT GAT GGG TCT TGG TCA Leu Met Gly Phe Asp Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser 300 305 310 315	962
GGA TCA CAG CCA TCC TGT GTT AAA GTG GCA GAC AGA GAG GTC GAC TGT Gly Ser Gln Pro Ser Cys Val Lys Val Ala Asp Arg Glu Val Asp Cys 320 325 330	1010
GAC AGT AAA GCT GTA GAC TTC TTG GAT GAT GTT GGT GAA CCT GTC AGG Asp Ser Lys Ala Val Asp Phe Leu Asp Val Gly Glu Pro Val Arg 335 340 345	1058
ATC CAC TGT CCT GCT GGC TGT TCT TTG ACA GCT GGT ACT GTG TGG GGT Ile His Cys Pro Ala Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly 350 355 360	1106
ACA GCC ATA TAC CAT GAA CTT TCC TCA GTG TGT CGT GCA GCC ATC CAT Thr Ala Ile Tyr His Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His 365 370 375	1154
GCT GGC AAG CTT CCA AAC TCT GGA GGA GCG GTG CAT GTT GTG AAC AAT Ala Gly Lys Leu Pro Asn Ser Gly Gly Ala Val His Val Val Asn Asn 380 385 390 395	1202
GGC CCC TAC TCG GAC TTT CTG GGT AGT GAC CTG AAT GGG ATA AAA TCG Gly Pro Tyr Ser Asp Phe Leu Gly Ser Asp Leu Asn Gly Ile Lys Ser 400 405 410	1250
GAA GAG TTG AAG TCT CTT GCC CGG AGT TTC CGA TTC GAT TAT GTC CGT Glu Glu Leu Lys Ser Leu Ala Arg Ser Phe Arg Phe Asp Tyr Val Arg 415 420 425	1298

CCT TTC TTG AGG TTG CCA CGG TTA CCC CTC CCA TTA GCT AGA GCA GCC Pro Phe Leu Arg Leu Pro Arg Leu Pro Leu Pro Leu Ala Arg Ala Ala 670 675 680	2066
AAA CCT CCT CCA AAA CCT AGA TCC TCA CAA CCC TCT ACT GTG GAC TTG Lys Pro Pro Pro Lys Pro Arg Ser Ser Gln Pro Ser Thr Val Asp Leu 685 690 695	2114
GCT TCT AAA GTT AAA CTA CCT GAA GGT CAT TAC CGG GTA GGG TCT CGA Ala Ser Lys Val Lys Leu Pro Glu Gly His Tyr Arg Val Gly Ser Arg 700 705 710 715	2162
GCC ATC TAC ACG TGC GAG TCG AGA TAC TAC GAA CTA CTT GGA TCT CAA Ala Ile Tyr Thr Cys Glu Ser Arg Tyr Tyr Glu Leu Leu Gly Ser Gln 720 725 730	2210
GGC AGA AGA TGT GAC TCT AAT GGA AAC TGG AGT GGT CGG CCA GCG AGC Gly Arg Arg Cys Asp Ser Asn Gly Asn Trp Ser Gly Arg Pro Ala Ser 735 740 745	2258
TGT ATT CCA GTT TGT GGA CGG TCA GAC TCT CCT CGT TCT CCT TTT ATC Cys Ile Pro Val Cys Gly Arg Ser Asp Ser Pro Arg Ser Pro Phe Ile 750 755 760	2306
TGG AAT GGG AAT TCT ACA GAA ATA GGT CAG TGG CCG TGG CAG GCA GGA Trp Asn Gly Asn Ser Thr Glu Ile Gly Gln Trp Pro Trp Gln Ala Gly 765 770 775	2354
ATC TCT AGA TGG CTT GCA GAC CAC AAT ATG TGG TTT CTC CAG TGT GGA Ile Ser Arg Trp Leu Ala Asp His Asn Met Trp Phe Leu Gln Cys Gly 780 785 790 795	2402
GGA TCT CTA TTG AAT GAG AAA TGG ATC GTC ACT GCT GCC CAC TGT GTC Gly Ser Leu Leu Asn Glu Lys Trp Ile Val Thr Ala Ala His Cys Val 800 805 810	2450
ACC TAC TCT GCT ACT GCT GAG ATT ATT GAC CCC AAT CAG TTT AAA ATG Thr Tyr Ser Ala Thr Ala Glu Ile Ile Asp Pro Asn Gln Phe Lys Met 815 820 825	2498
TAT CTG GGC AAG TAC TAC CGT GAT GAC AGT AGA GAC GAT GAC TAT GTA Tyr Leu Gly Lys Tyr Tyr Arg Asp Asp Ser Arg Asp Asp Asp Tyr Val 830 835 840	2546
CAA GTA AGA GAG GCT CTT GAG ATC CAC GTG AAT CCT AAC TAC GAC CCC Gln Val Arg Glu Ala Leu Glu Ile His Val Asn Pro Asn Tyr Asp Pro 845 850 855	2594
GGC AAT CTC AAC TTT GAC ATA GCC CTA ATT CAA CTG AAA ACT CCT GTT Gly Asn Leu Asn Phe Asp Ile Ala Leu Ile Gln Leu Lys Thr Pro Val 860 865 870 875	2642
ACT TTG ACA ACA CGA GTC CAA CCA ATC TGT CTG CCT ACT GAC ATC ACA Thr Leu Thr Thr Arg Val Gln Pro Ile Cys Leu Pro Thr Asp Ile Thr 880 885 890	2690
ACA AGA GAA CAC TTG AAG GAG GGA ACA TTA GCA GTG GTG ACA GGT TGG Thr Arg Glu His Leu Lys Glu Gly Thr Leu Ala Val Val Thr Gly Trp 895 900 905	2738

GGT TTG AAT GAA AAC AAC ACC TAT TCA GAG ACG ATT CAA CAA GCT GTG Gly Leu Asn Glu Asn Asn Thr Tyr Ser Glu Thr Ile Gln Gln Ala Val 910 915 920	2786
CTA CCT GTT GTT GCA GCC AGC ACC TGT GAA GAG GGG TAC AAG GAA GCA Leu Pro Val Val Ala Ala Ser Thr Cys Glu Glu Gly Tyr Lys Glu Ala 925 930 935	2834
GAC TTA CCA CTG ACA GTA ACA GAG AAC ATG TTC TGT GCA GGT TAC AAG Asp Leu Pro Leu Thr Val Thr Glu Asn Met Phe Cys Ala Gly Tyr Lys 940 945 950 955	2882
AAG GGA CGT TAT GAT GCC TGC AGT GGG GAC AGT GGA GGA CCT TTA GTG Lys Gly Arg Tyr Asp Ala Cys Ser Gly Asp Ser Gly Gly Pro Leu Val 960 965 970	2930
TTT GCT GAT GAT TCC CGT ACC GAA AGG CGG TGG GTC TTG GAA GGG ATT Phe Ala Asp Asp Ser Arg Thr Glu Arg Arg Trp Val Leu Glu Gly Ile 975 980 985	2978
GTC AGC TGG GGC AGT CCC AGT GGA TGT GGC AAG GCG AAC CAG TAC GGG Val Ser Trp Gly Ser Pro Ser Gly Cys Gly Lys Ala Asn Gln Tyr Gly 990 995 1000	3026
GGC TTC ACT AAA GTT AAC GTT TTC CTG TCA TGG ATT AGG CAG TTC ATT Gly Phe Thr Lys Val Asn Val Phe Leu Ser Trp Ile Arg Gln Phe Ile 1005 1010 1015	3074
TGAAACTGAT CTAAATATTT TAAGCATGGT TATAAACGTC TTGTTTCCTA TTATTGCTTT	3134
ACTAGTTTAA CCCATAAGAA GGTTAACTGG GTAAGGCACA AGGATCATTG TTTCTGTTTG	3194
TTTTTACAAA TGGTTATTTT AGTCAGTGAA TGAGAATAGT ATCCATTGAA GACTGTTACC	3254
TTTTATTCTA CCTTTTATA TTACTATGTA AGTATTGGG ATATCTTCTA CACATGAAAA	3314
TTCTGTCATT TTACCATAAA TTTGGTTTCT GGTGTGTGCT AAGTCCACCA GTAGAGAACG	3374
ATGTAATTTT CACTAGCACA TGAAATAAAT ATAGAACAAA TCTATTATAA ACTACCTTAA	3434
AAAAAAAAAAAA AAAA	3448

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1019 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Val Leu Ala Ser Phe Leu Val Ser Gly Leu Val Leu Gly Leu Leu
1 5 10 15

Ala Gln Lys Met Arg Pro Val Gln Ser Lys Gly Val Asp Leu Gly Leu
20 25 30

Cys	Asp	Glu	Thr	Arg	Phe	Glu	Cys	Lys	Cys	Gly	Asp	Pro	Gly	Tyr	Val	
		35						40				45				
Phe	Asn	Ile	Pro	Val	Lys	Gln	Cys	Thr	Tyr	Phe	Tyr	Arg	Trp	Arg	Pro	
	50					55					60					
Tyr	Cys	Lys	Pro	Cys	Asp	Asp	Leu	Glu	Ala	Lys	Asp	Ile	Cys	Pro	Lys	
65					70					75					80	
Tyr	Lys	Arg	Cys	Gln	Glu	Cys	Lys	Ala	Gly	Leu	Asp	Ser	Cys	Val	Thr	
				85					90					95		
Cys	Pro	Pro	Asn	Lys	Tyr	Gly	Thr	Trp	Cys	Ser	Gly	Glu	Cys	Gln	Cys	
			100					105					110			
Lys	Asn	Gly	Gly	Ile	Cys	Asp	Gln	Arg	Thr	Gly	Ala	Cys	Ala	Cys	Arg	
		115					120					125				
Asp	Arg	Tyr	Glu	Gly	Val	His	Cys	Glu	Ile	Leu	Lys	Gly	Cys	Pro	Leu	
	130					135					140					
Leu	Pro	Ser	Asp	Ser	Gln	Val	Gln	Glu	Val	Arg	Asn	Pro	Pro	Asp	Asn	
145					150					155					160	
Pro	Gln	Thr	Ile	Asp	Tyr	Ser	Cys	Ser	Pro	Gly	Phe	Lys	Leu	Lys	Gly	
				165					170					175		
Met	Ala	Arg	Ile	Ser	Cys	Leu	Pro	Asn	Gly	Gln	Trp	Ser	Asn	Phe	Pro	
			180					185					190			
Pro	Lys	Cys	Ile	Arg	Glu	Cys	Ala	Met	Val	Ser	Ser	Pro	Glu	His	Gly	
		195					200					205				
Lys	Val	Asn	Ala	Leu	Ser	Gly	Asp	Met	Ile	Glu	Gly	Ala	Thr	Leu	Arg	
	210					215					220					
Phe	Ser	Cys	Asp	Ser	Pro	Tyr	Tyr	Leu	Ile	Gly	Gln	Glu	Thr	Leu	Thr	
225					230					235					240	
Cys	Gln	Gly	Asn	Gly	Gln	Trp	Asn	Gly	Gln	Ile	Pro	Gln	Cys	Lys	Asn	
				245					250					255		
Leu	Val	Phe	Cys	Pro	Asp	Leu	Asp	Pro	Val	Asn	His	Ala	Glu	His	Lys	
			260					265					270			
Val	Lys	Ile	Gly	Val	Glu	Gln	Lys	Tyr	Gly	Gln	Phe	Pro	Gln	Gly	Thr	
		275					280					285				
Glu	Val	Thr	Tyr	Thr	Cys	Ser	Gly	Asn	Tyr	Phe	Leu	Met	Gly	Phe	Asp	
	290					295					300					
Thr	Leu	Lys	Cys	Asn	Pro	Asp	Gly	Ser	Trp	Ser	Gly	Ser	Gln	Pro	Ser	
305					310					315					320	
Cys	Val	Lys	Val	Ala	Asp	Arg	Glu	Val	Asp	Cys	Asp	Ser	Lys	Ala	Val	
				325					330					335		
Asp	Phe	Leu	Asp	Asp	Val	Gly	Glu	Pro	Val	Arg	Ile	His	Cys	Pro	Ala	
				340				345					350			

Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly Thr Ala Ile Tyr His
 355 360 365
 Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His Ala Gly Lys Leu Pro
 370 375 380
 Asn Ser Gly Gly Ala Val His Val Val Asn Asn Gly Pro Tyr Ser Asp
 385 390 395 400
 Phe Leu Gly Ser Asp Leu Asn Gly Ile Lys Ser Glu Glu Leu Lys Ser
 405 410 415
 Leu Ala Arg Ser Phe Arg Phe Asp Tyr Val Arg Ser Ser Thr Ala Gly
 420 425 430
 Lys Ser Gly Cys Pro Asp Gly Trp Phe Glu Val Asp Glu Asn Cys Val
 435 440 445
 Tyr Val Thr Ser Lys Gln Arg Ala Trp Glu Arg Ala Gln Gly Val Cys
 450 455 460
 Thr Asn Met Ala Ala Arg Leu Ala Val Leu Asp Lys Asp Val Ile Pro
 465 470 475 480
 Asn Ser Leu Thr Glu Thr Leu Arg Gly Lys Gly Leu Thr Thr Thr Trp
 485 490 495
 Ile Gly Leu His Arg Leu Asp Ala Glu Lys Pro Phe Ile Trp Glu Leu
 500 505 510
 Met Asp Arg Ser Asn Val Val Leu Asn Asp Asn Leu Thr Phe Trp Ala
 515 520 525
 Ser Gly Glu Pro Gly Asn Glu Thr Asn Cys Val Tyr Met Asp Ile Gln
 530 535 540
 Asp Gln Leu Gln Ser Val Trp Lys Thr Lys Ser Cys Phe Gln Pro Ser
 545 550 555 560
 Ser Phe Ala Cys Met Met Asp Leu Ser Asp Arg Asn Lys Ala Lys Cys
 565 570 575
 Asp Asp Pro Gly Ser Leu Glu Asn Gly His Ala Thr Leu His Gly Gln
 580 585 590
 Ser Ile Asp Gly Phe Tyr Ala Gly Ser Ser Ile Arg Tyr Ser Cys Glu
 595 600 605
 Val Leu His Tyr Leu Ser Gly Thr Glu Thr Val Thr Cys Thr Thr Asn
 610 615 620
 Gly Thr Trp Ser Ala Pro Lys Pro Arg Cys Ile Lys Val Ile Thr Cys
 625 630 635 640
 Gln Asn Pro Pro Val Pro Ser Tyr Gly Ser Val Glu Ile Lys Pro Pro
 645 650 655
 Ser Arg Thr Asn Ser Ile Ser Arg Val Gly Ser Pro Phe Leu Arg Leu
 660 665 670

Pro Arg Leu Pro Leu Pro Leu Ala Arg Ala Ala Lys Pro Pro Pro Lys
 675 680 685
 Pro Arg Ser Ser Gln Pro Ser Thr Val Asp Leu Ala Ser Lys Val Lys
 690 695 700
 Leu Pro Glu Gly His Tyr Arg Val Gly Ser Arg Ala Ile Tyr Thr Cys
 705 710 715 720
 Glu Ser Arg Tyr Tyr Glu Leu Leu Gly Ser Gln Gly Arg Arg Cys Asp
 725 730 735
 Ser Asn Gly Asn Trp Ser Gly Arg Pro Ala Ser Cys Ile Pro Val Cys
 740 745 750
 Gly Arg Ser Asp Ser Pro Arg Ser Pro Phe Ile Trp Asn Gly Asn Ser
 755 760 765
 Thr Glu Ile Gly Gln Trp Pro Trp Gln Ala Gly Ile Ser Arg Trp Leu
 770 775 780
 Ala Asp His Asn Met Trp Phe Leu Gln Cys Gly Gly Ser Leu Leu Asn
 785 790 795 800
 Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Thr Tyr Ser Ala Thr
 805 810 815
 Ala Glu Ile Ile Asp Pro Asn Gln Phe Lys Met Tyr Leu Gly Lys Tyr
 820 825 830
 Tyr Arg Asp Asp Ser Arg Asp Asp Asp Tyr Val Gln Val Arg Glu Ala
 835 840 845
 Leu Glu Ile His Val Asn Pro Asn Tyr Asp Pro Gly Asn Leu Asn Phe
 850 855 860
 Asp Ile Ala Leu Ile Gln Leu Lys Thr Pro Val Thr Leu Thr Thr Arg
 865 870 875 880
 Val Gln Pro Ile Cys Leu Pro Thr Asp Ile Thr Thr Arg Glu His Leu
 885 890 895
 Lys Glu Gly Thr Leu Ala Val Val Thr Gly Trp Gly Leu Asn Glu Asn
 900 905 910
 Asn Thr Tyr Ser Glu Thr Ile Gln Gln Ala Val Leu Pro Val Val Ala
 915 920 925
 Ala Ser Thr Cys Glu Glu Gly Tyr Lys Glu Ala Asp Leu Pro Leu Thr
 930 935 940
 Val Thr Glu Asn Met Phe Cys Ala Gly Tyr Lys Lys Gly Arg Tyr Asp
 945 950 955 960
 Ala Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Phe Ala Asp Asp Ser
 965 970 975
 Arg Thr Glu Arg Arg Trp Val Leu Glu Gly Ile Val Ser Trp Gly Ser
 980 985 990

Pro Ser Gly Cys Gly Lys Ala Asn Gln Tyr Gly Gly Phe Thr Lys Val
995 1000 1005

Asn Val Phe Leu Ser Trp Ile Arg Gln Phe Ile
1010 1015

CLAIMS:

What is claimed is:

1. A recombinant DNA vector comprising a baculovirus-derived vector and a cDNA encoding a Factor C enzyme.
2. The vector of claim 1, wherein said baculovirus-derived vector is pFastBacI.
3. The vector of claim 2, that is pFastBac/CrFC21.
4. The vector of claim 1, wherein said cDNA encoding a Factor C enzyme comprises a polynucleotide that hybridizes to a nucleotide sequence selected from SEQ ID NO:1, the complement of SEQ ID NO:1, SEQ ID NO:3, the complement of SEQ ID NO:3 or a cDNA from *Tachypleus tridentatus* encoding a Factor C enzyme, under
5 stringent conditions.
5. The vector of claim 1, wherein said cDNA encoding a Factor C enzyme comprises a polynucleotide that encodes an amino acid sequence selected from SEQ ID NO:2, SEQ ID NO:4, or the amino acid sequence of Factor C from *Tachypleus tridentatus*.
6. The vector of claim 1, wherein said cDNA encoding a Factor C enzyme comprises a polynucleotide having the sequence of SEQ. ID. NO. 1 or SEQ ID NO:3.

7. An assay for endotoxin comprising:
 - i) contacting a sample to be assayed, comprising a peptide cleavable to produce a chromogenic or fluorogenic moiety, with a recombinant Factor C of a horseshoe crab; and
 - 5 ii) measuring the amount of said chromogenic or fluorogenic moiety cleaved from said peptide.
8. The assay of claim 7, wherein said peptide is N-t-Boc-Val-Pro-Arg-MCA, Mu-Val-Pro-Arg-AFC or Boc-Val-Pro-Arg-pNA.
9. The assay of claim 7, wherein said recombinant Factor C is obtained from an insect host culture.
10. The assay of claim 8, wherein said recombinant Factor C is obtained from an insect host cell culture.
11. The assay of claim 9, wherein said insect host cell is a lepidopteran cell.
12. The assay of claim 10, wherein said insect host cell is a lepidopteran cell.
13. An assay for endotoxin comprising:
 - i) contacting a sample to be assayed with an immobilized antibody that specifically binds to lipopolysaccharide or an immobilized antibody that specifically binds to lipid A to form a complex between said antibody and endotoxin in said sample;

- 5 ii) contacting said complex with recombinant Factor C of a horseshoe crab
to form immobilized Factor C; and
- iii) contacting said immobilized Factor C with an antibody that specifically
binds to said immobilized Factor C; and
- iv) quantitating the amount of said antibody specifically bound to said
10 immobilized Factor C.

14. The assay of claim 13, wherein said recombinant Factor C is obtained
from an insect host cell culture.

15. The assay of claim 14, wherein said insect host cell is a lepidopteran cell.

16. An assay for endotoxin comprising:

i) contacting a sample to be assayed, comprising a peptide cleavable to
produce a chromogenic or fluorogenic moiety, with a recombinant Factor C of a
horseshoe crab; and

ii) measuring the amount of said chromogenic or fluorogenic moiety
cleaved from said peptide

wherein said recombinant Factor C is produced using a vector of claim 1.

17. An assay for endotoxin comprising:

i) contacting a sample to be assayed with an immobilized antibody that
specifically binds to lipopolysaccharide or an antibody that specifically binds to lipid A
to form a complex between said antibody and endotoxin in said sample;

- 5 ii) contacting said complex with recombinant Factor C of a horseshoe crab
to form immobilized Factor C; and
- iii) contacting said immobilized Factor C with an antibody that specifically
binds to said immobilized Factor C; and
- iv) quantitating the amount of said antibody specifically bound to said
10 immobilized Factor C;

wherein said recombinant Factor C is produced using a vector of claim 1.

18. A method for removing endotoxin or lipid A from a sample comprising:

- i) contacting immobilized recombinant Factor C with said sample, so that
endotoxin or lipid A in said sample binds to said immobilized recombinant Factor C; and
- ii) separating said immobilized recombinant Factor C, having said
5 endotoxin or lipid A bound thereto, from said sample;

wherein said recombinant Factor C is produced using a vector of claim 1.

1/16

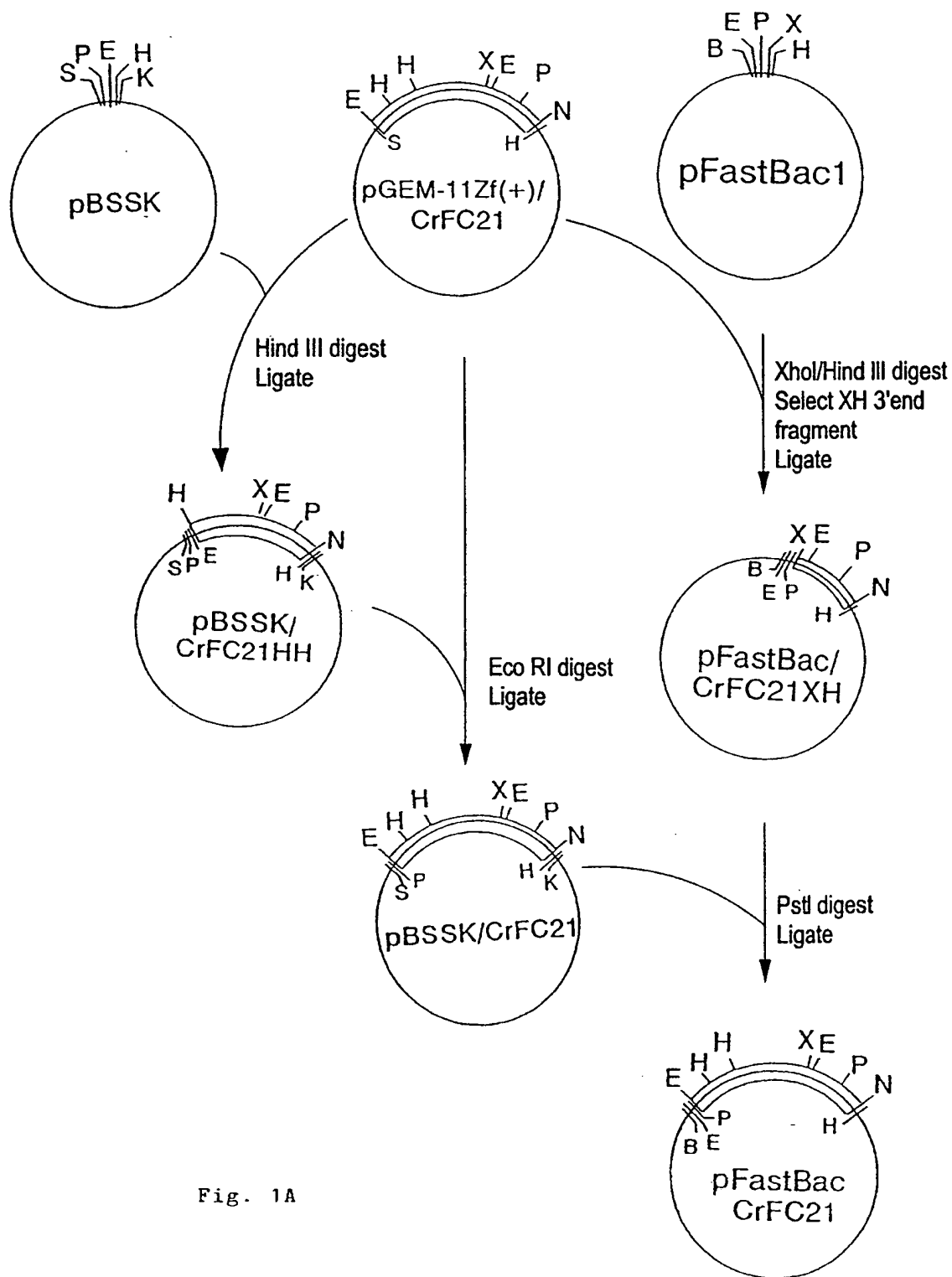


Fig. 1A

2/16

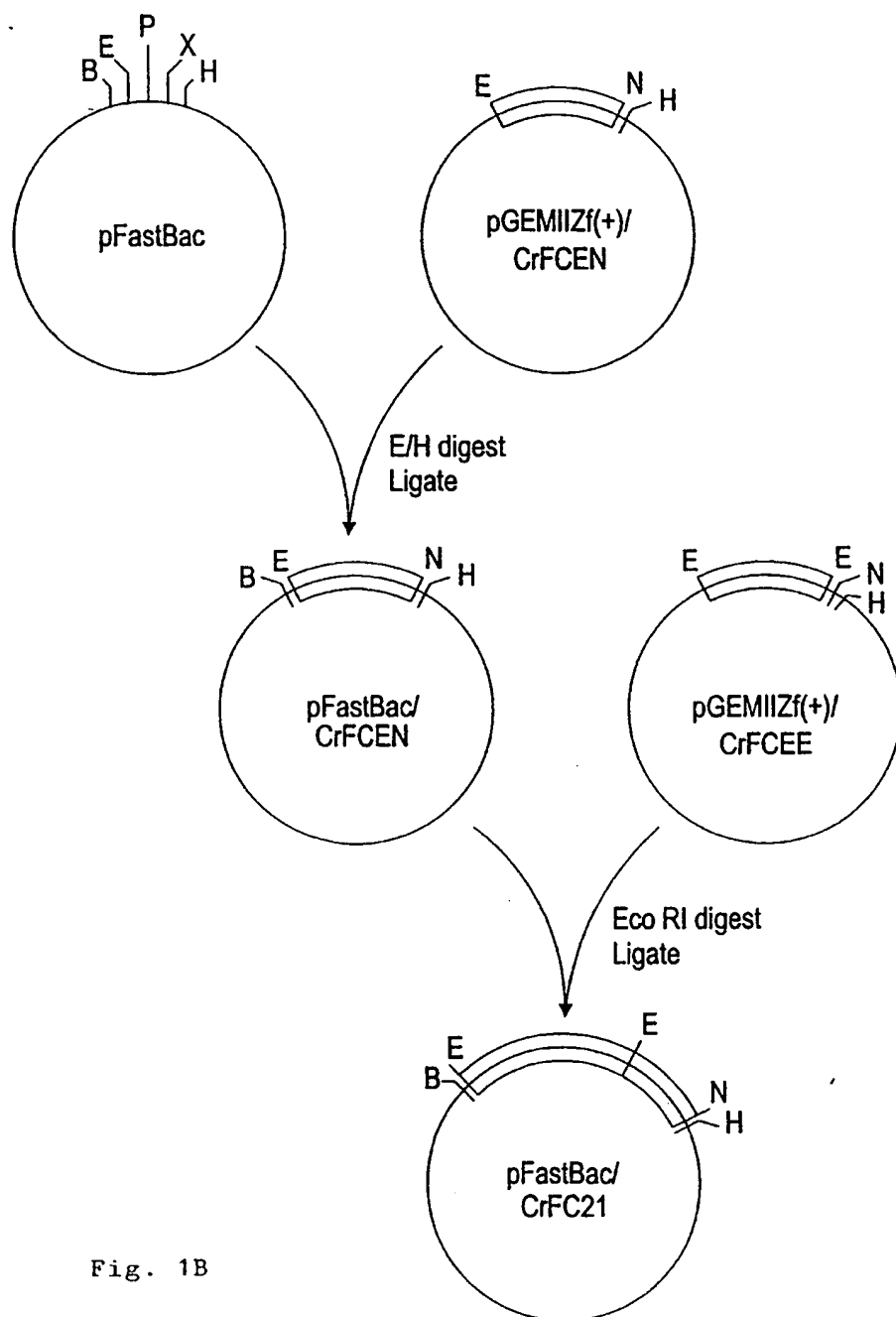


Fig. 1B

3/16

24 h p.i. 48 h p.i.
(Reducing SDS-PAGE) (Reducing SDS-PAGE)

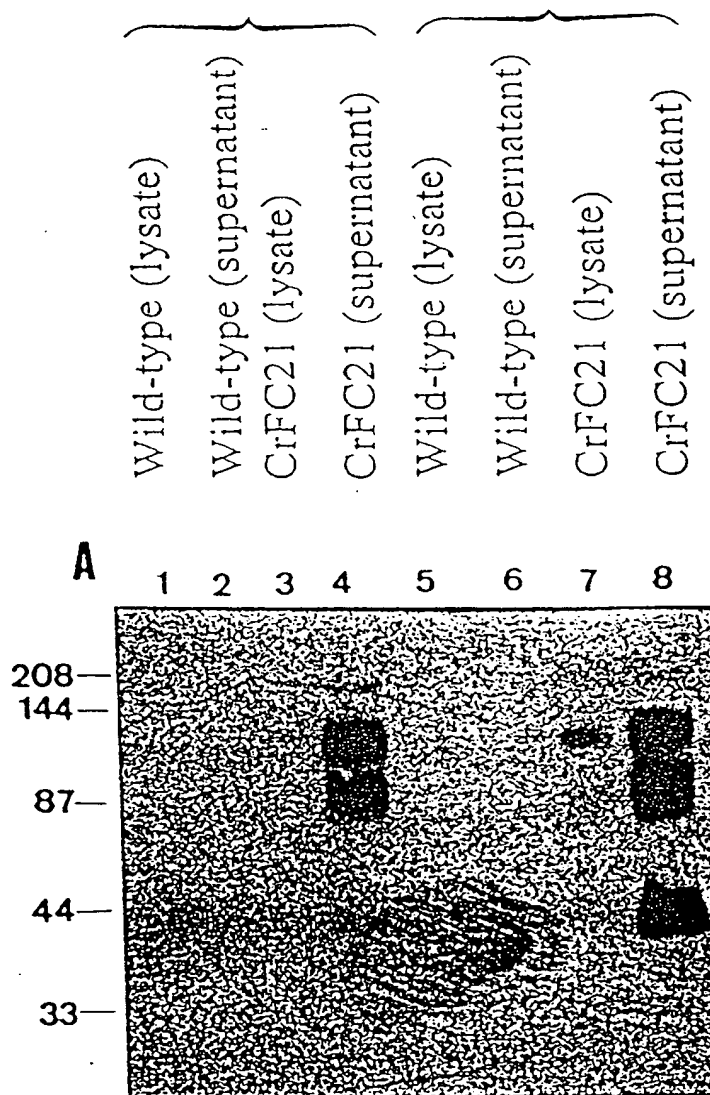


Fig. 2A

BEST AVAILABLE COPY

4/16

72 h p.i. 72 h p.i.
(Reducing SDS-PAGE) (Non-reducing SDS-PAGE)

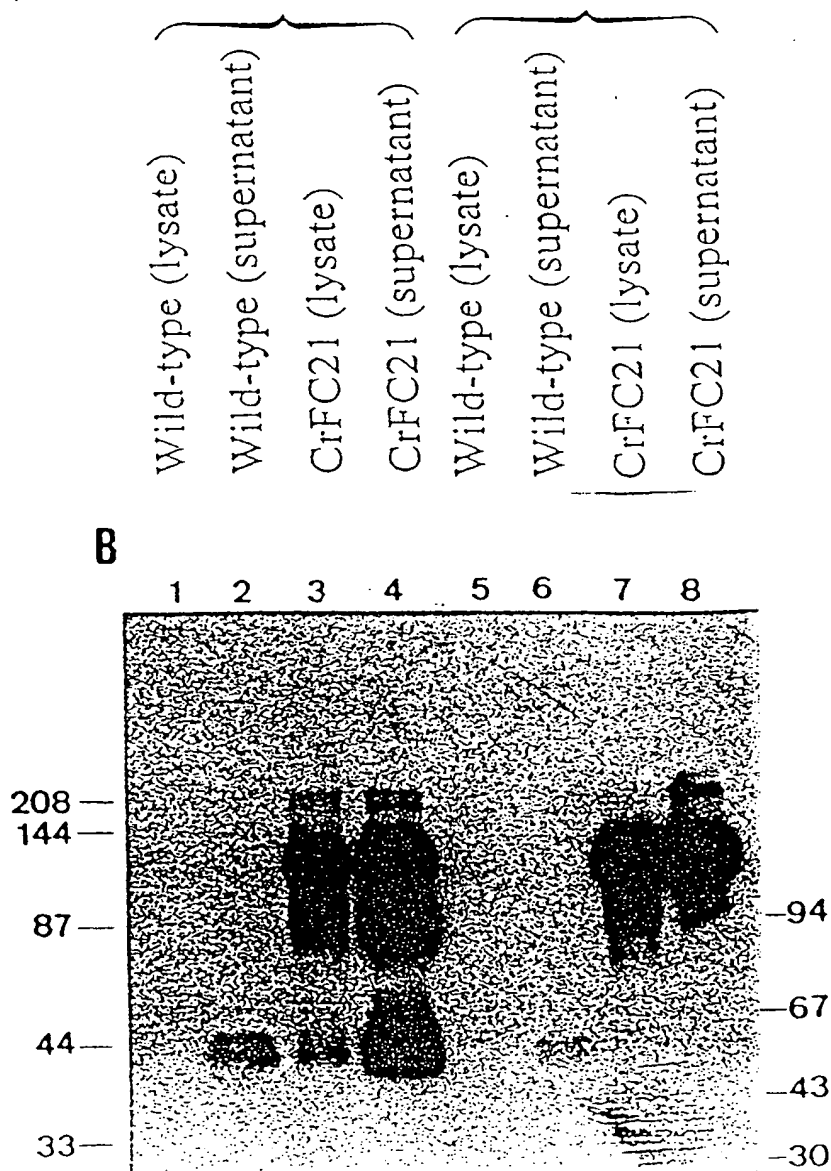


Fig. 2B

BEST AVAILABLE COPY

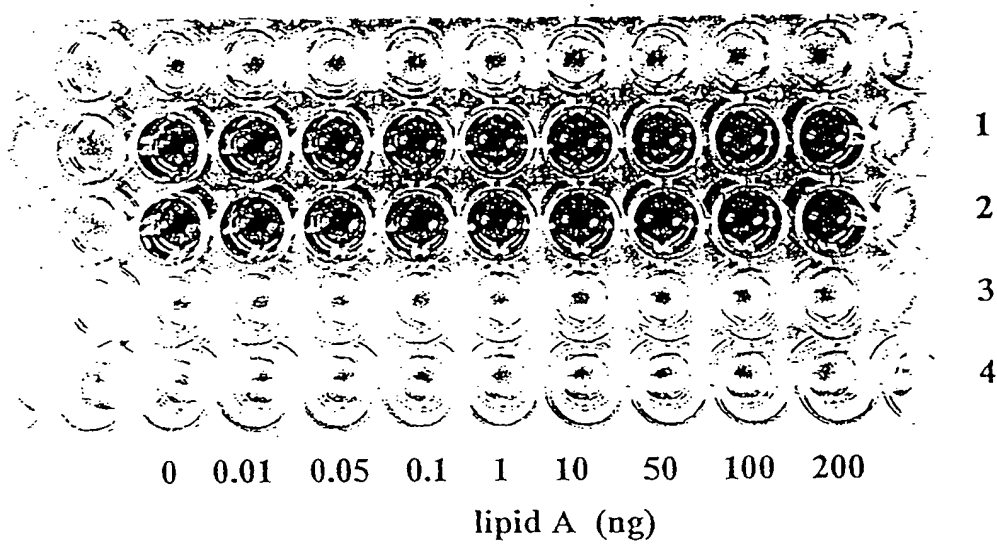
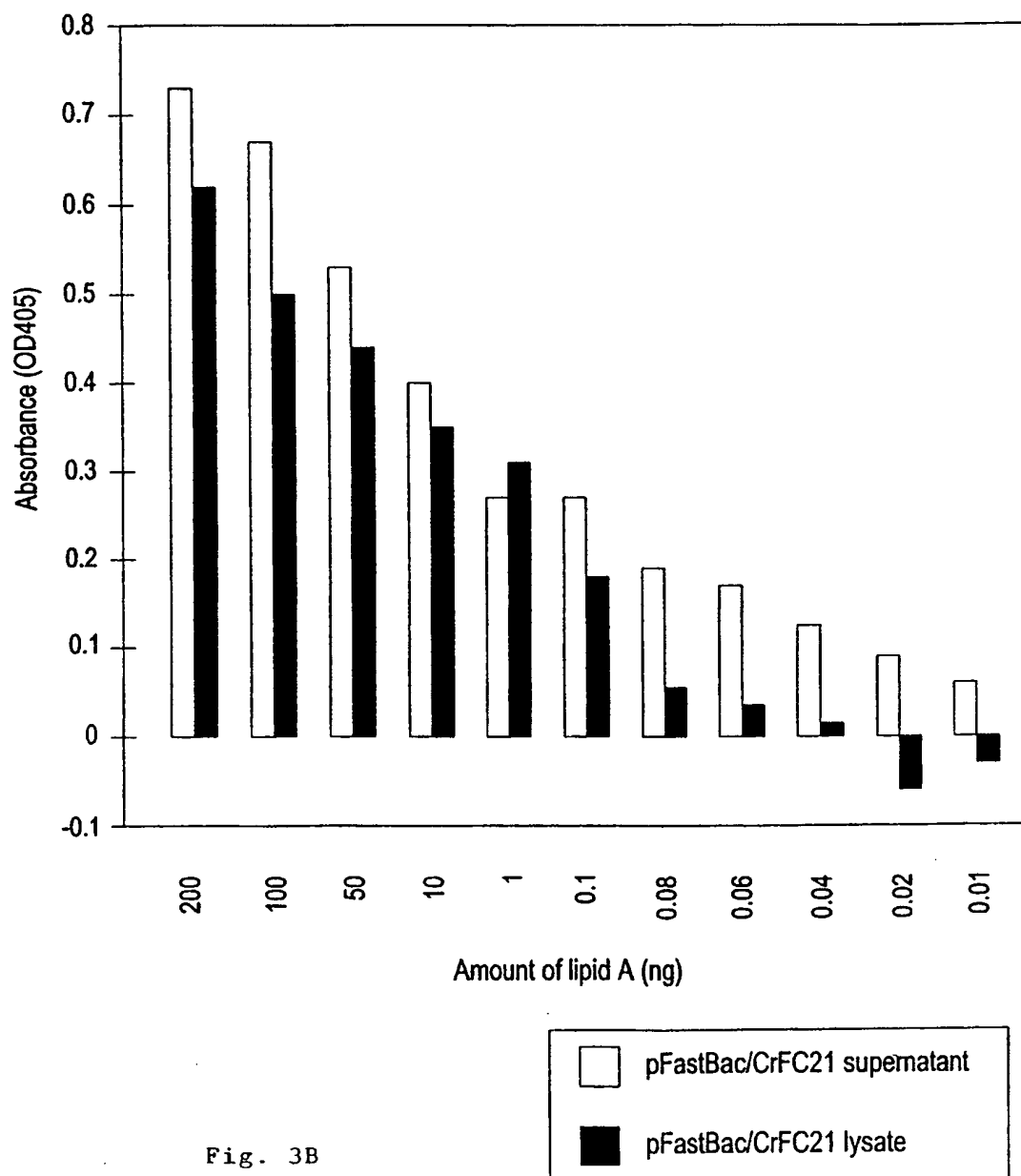


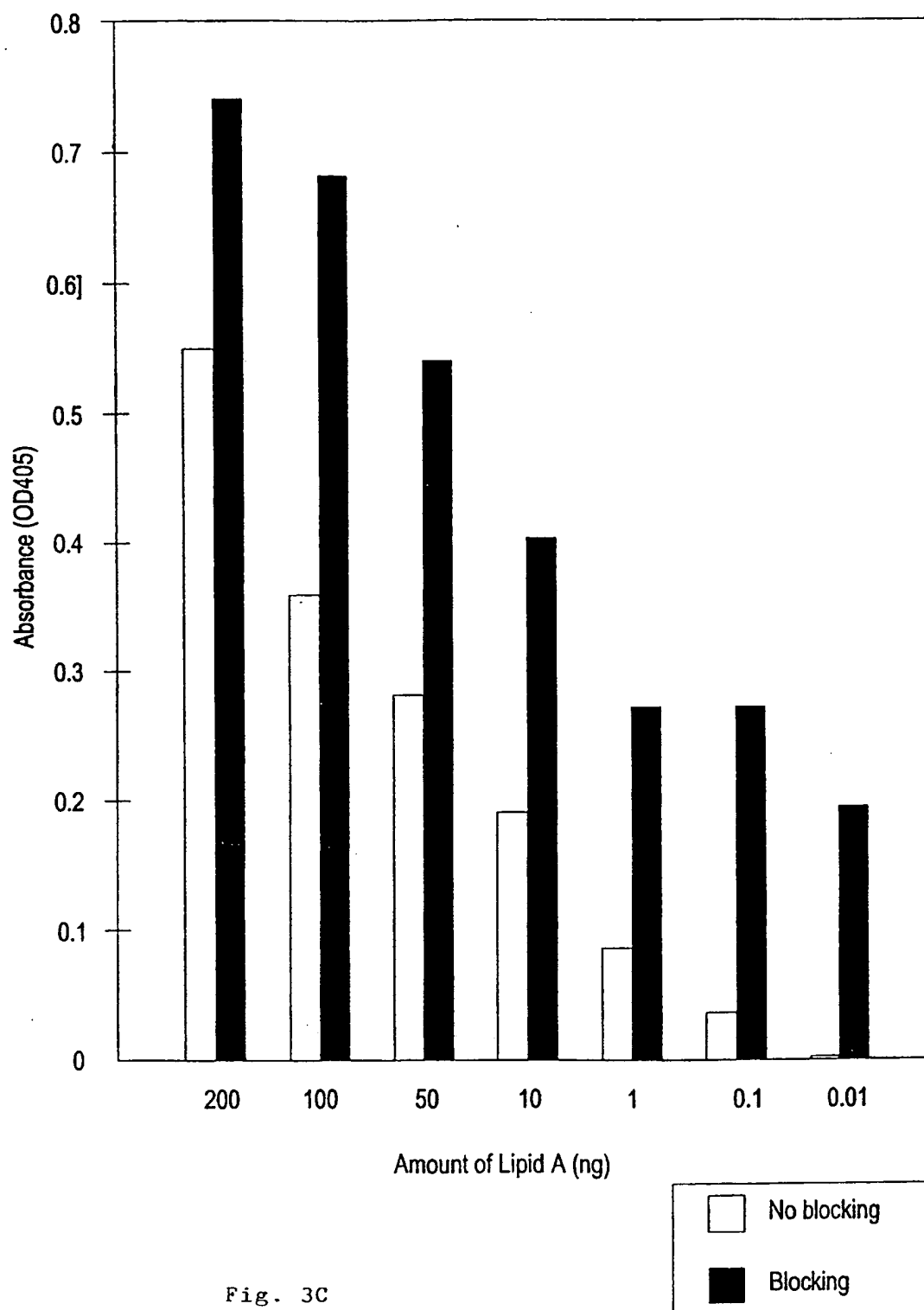
Fig. 3A

BEST AVAILABLE COPY

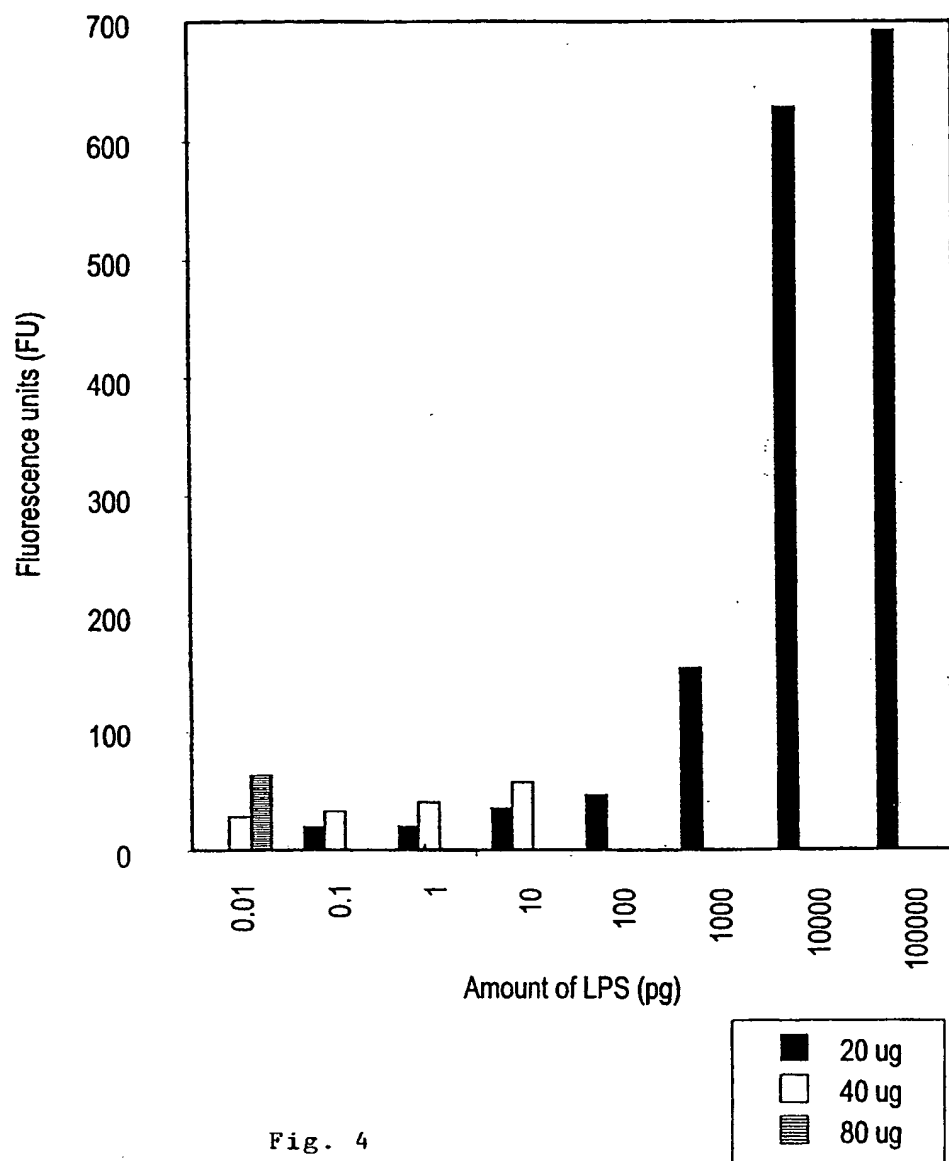
6/16



7/16



8/16



9/16

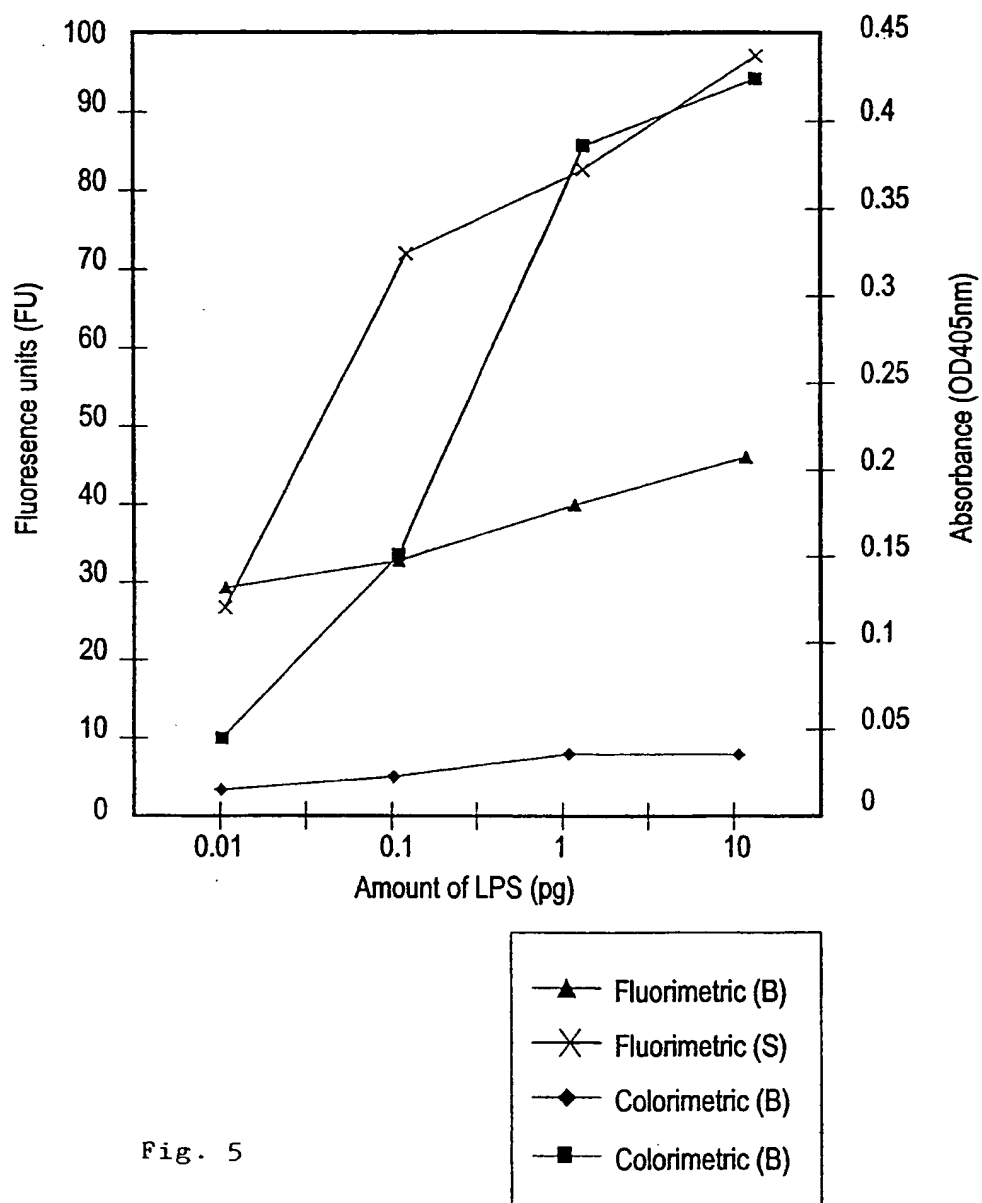
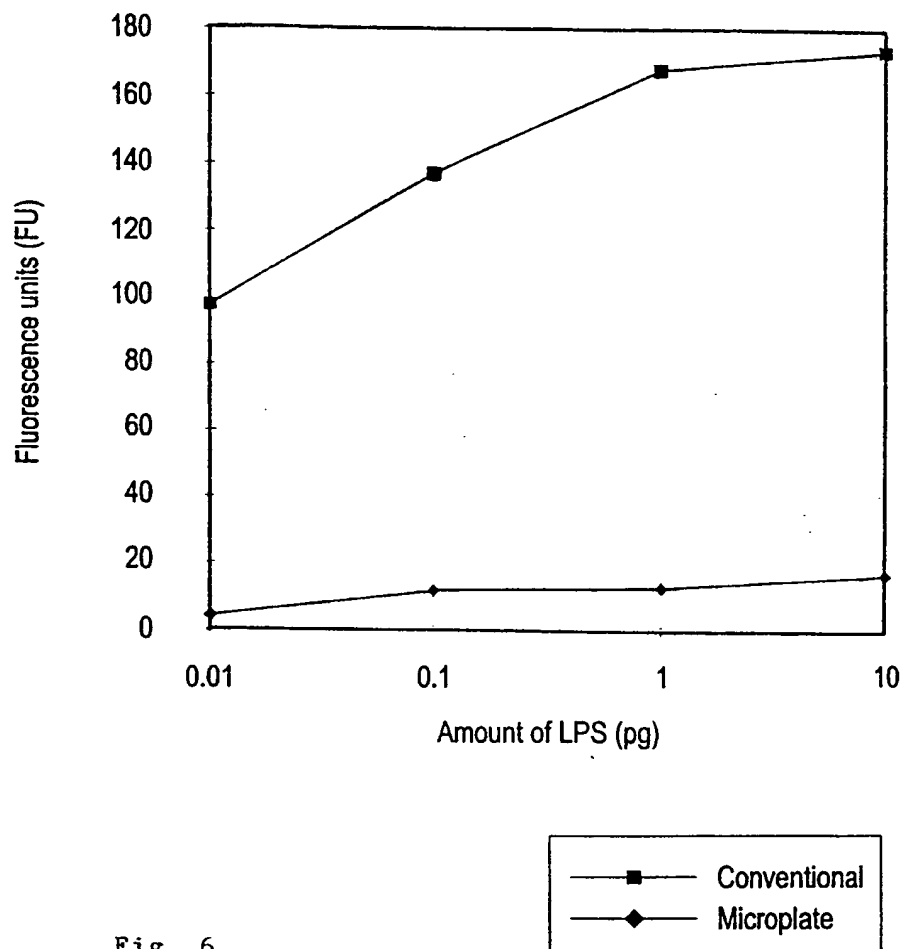
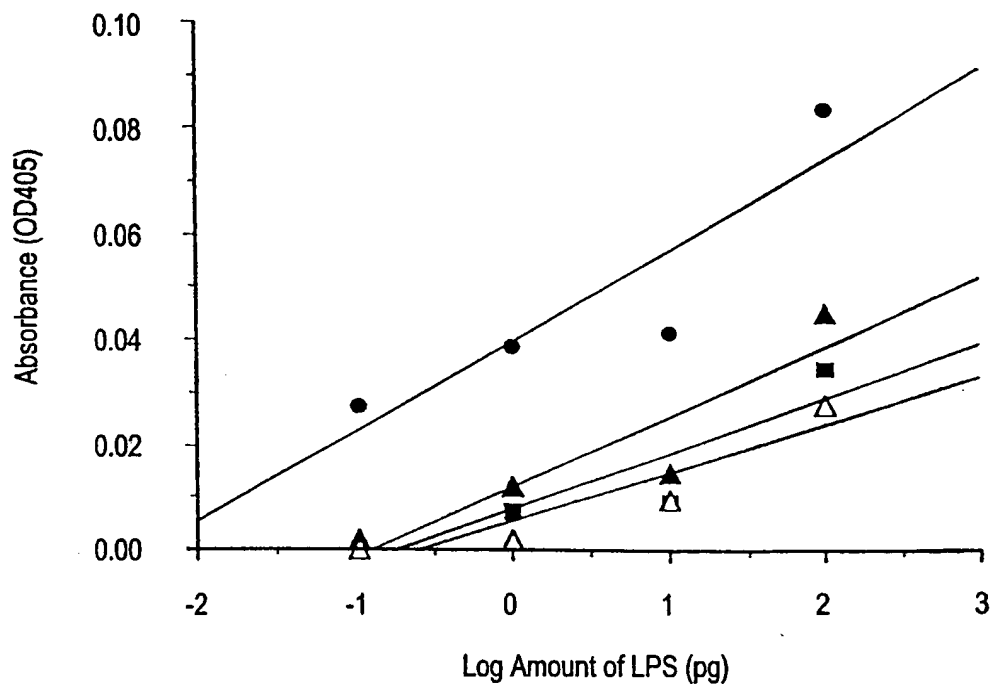


Fig. 5

10/16



11/16



$y = 3.9130e-2 + 1.7115e-2x$	$R^2 = 0.807$	●	100ug prot(2mM subs)
$y = 1.1760e-2 + 1.3355e-2x$	$R^2 = 0.843$	▲	100ug prot(4mM subs)
$y = 7.5000e-3 + 1.0500e-2x$	$R^2 = 0.814$	■	60ug prot(2mM subs)
$y = 5.3350e-3 + 9.1050e-3x$	$R^2 = 0.867$	△	60ug prot(4mM subs)

Fig. 7

12/16

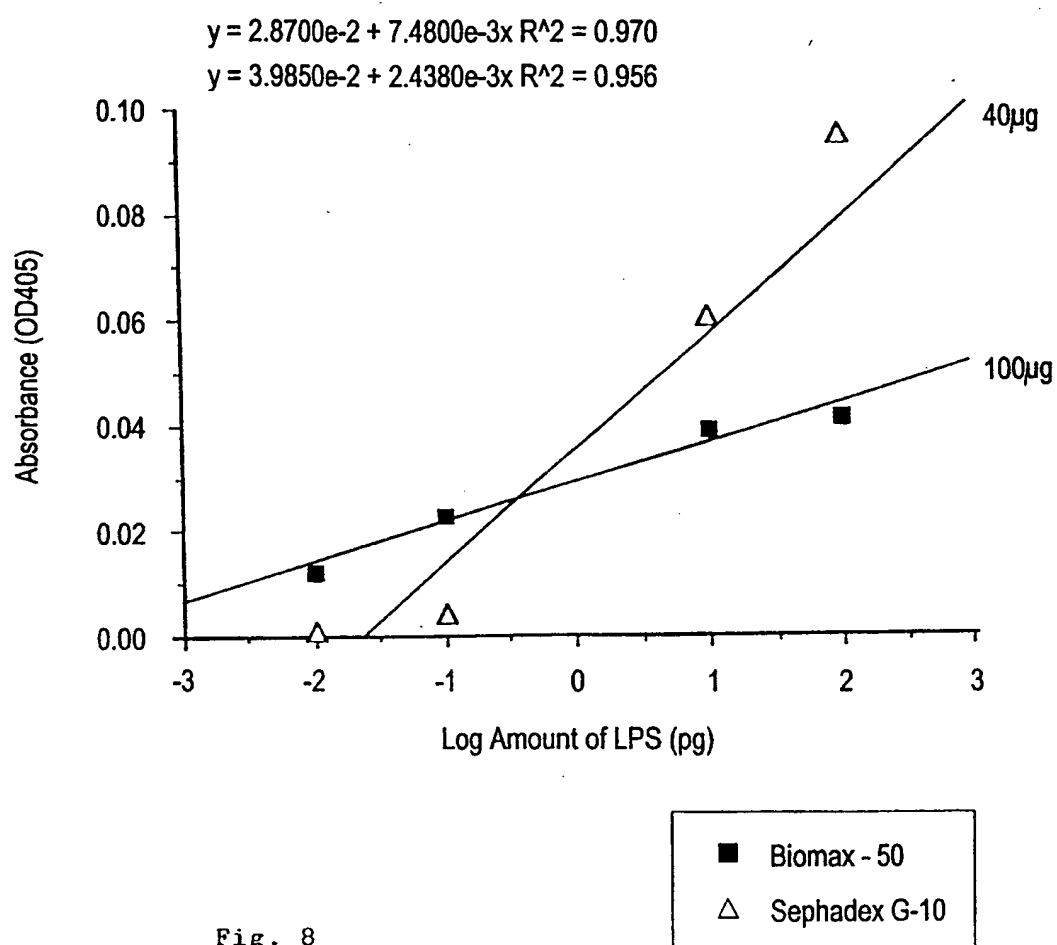
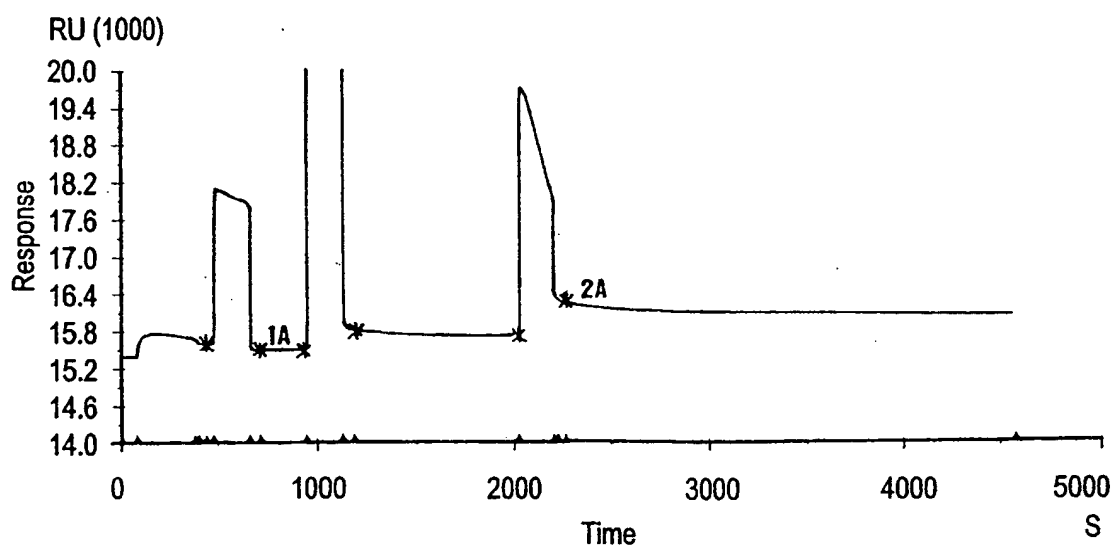


Fig. 8

13/16



Fc	Time	AbsResp	Slope	LRSD	Baseline	RelResp	Ld
1	445.5	15605.1	-1.30	0.86	No	#N/A	before w/t
1	713.5	15509.4	-3.51	4.15	No	#N/A	w/t
1	936.5	15484.9	-0.16	0.18	No	#N/A	before w/t2x
1	1197.5	15798.6	-1.62	0.26	No	#N/A	w/t2x
1	2023.5	15728.3	-0.46	0.82	No	#N/A	before rFCSf9
1	2268.5	16281.2	-3.28	0.62	No	#N/A	rFCSf9

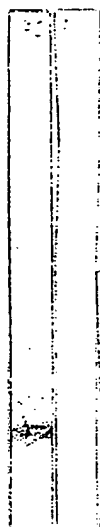
Fig. 9

14/16

E. coli (D31m4)

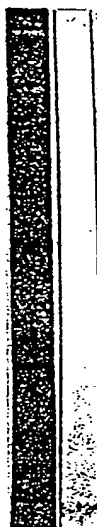
LPS

1 2

*K. pneumoniae*

LPS

1 2

*S. minnesota*

LPS

1 2

*S. minnesota*

Lipid A

1 2



Fig. 10

BEST AVAILABLE COPY

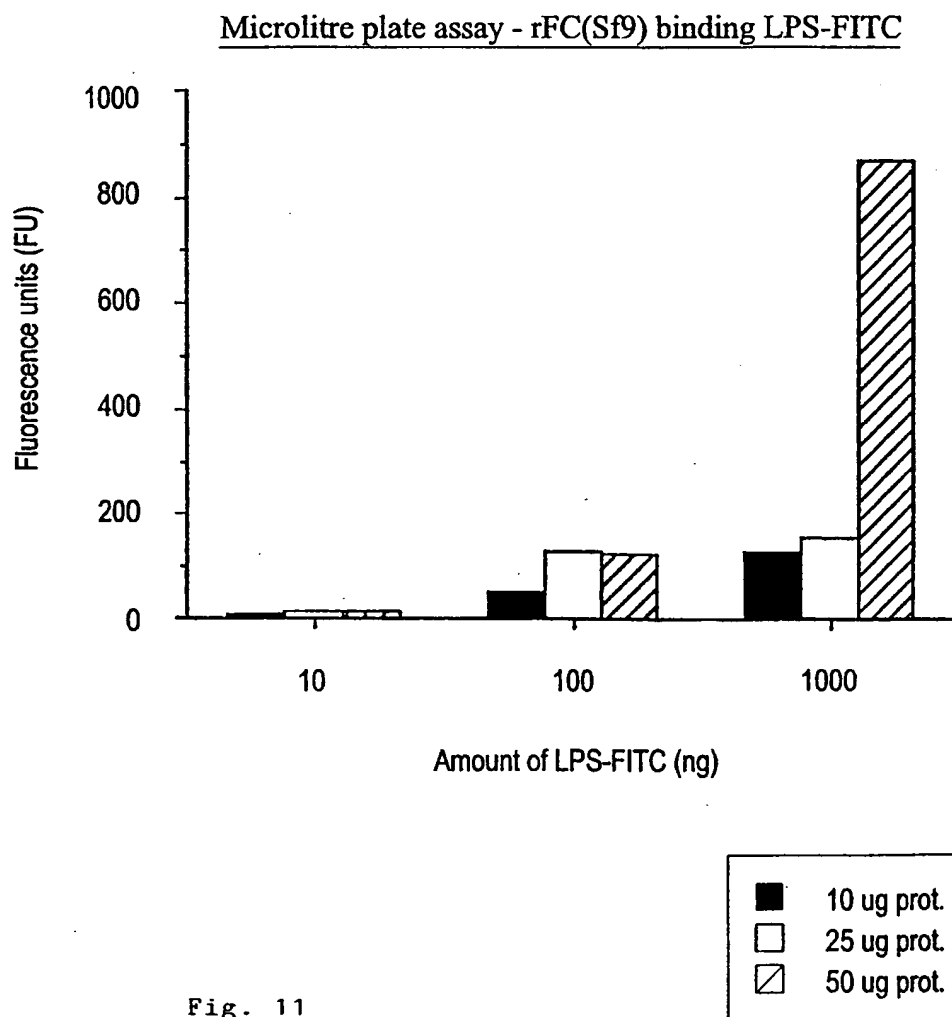


Fig. 11

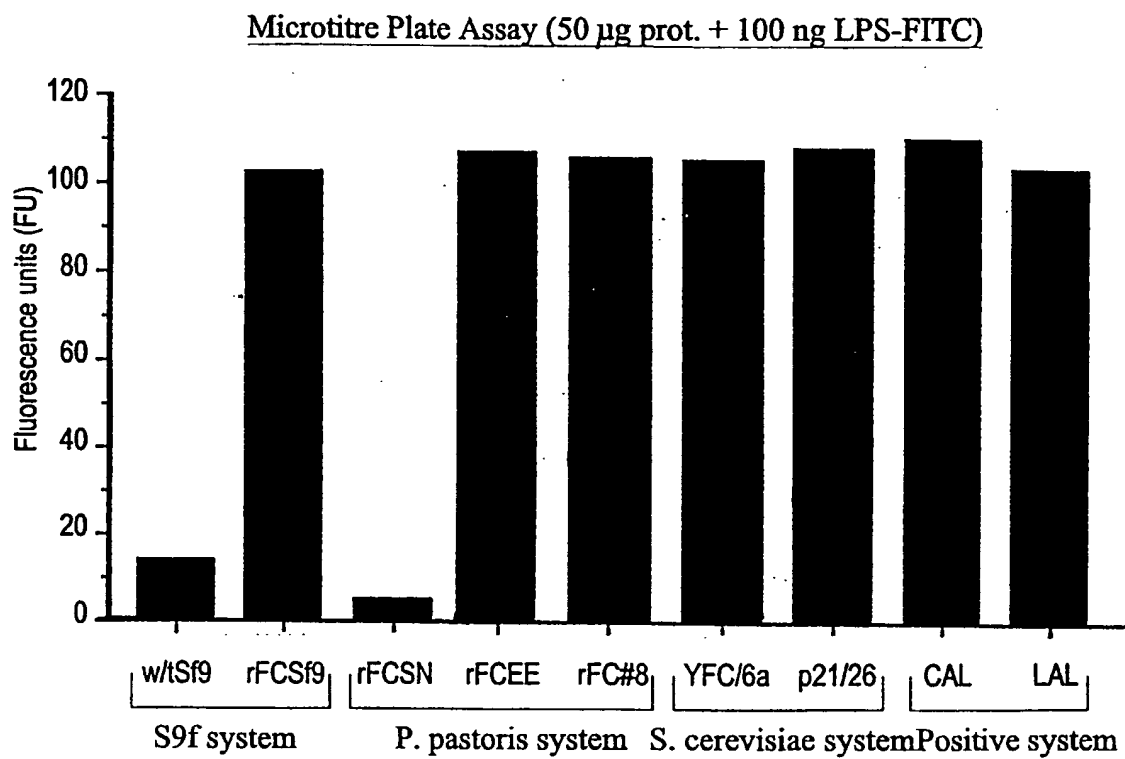


Fig. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG 98/00073

A. CLASSIFICATION OF SUBJECT MATTER IPC ⁶ : C 12 N 15/63, 15/55; C 12 Q 1/37 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC ⁶ : 15/63, 15/55; C 12 Q 1/37 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPI		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Database WPI, week 51, London: Derwent Publication Ltd. AN 97-557571, & SG 42 456 A1 (NATIONAL UNIVERSITY OF SINGAPORE) 15 August 1997 (15.08.97). -----	- 1
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 04 January 1999 (04.01.99)		Date of mailing of the international search report 18 January 1999 (18.01.99)
Name and mailing address of the ISA/ Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535		Authorized officer Wolf Telephone No. 1/53424/436